

# Evolutionary and Immunological Aspects on $\beta_2$ -microglobulin

Lennart Lögdberg

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EVOLUTIONARY AND IMMUNOLOGICAL ASPECTS ON  $\beta_2$ -MICROGLOBULIN

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 Evolutionary and immunological aspects on  $\beta_2$ -microglobulin

Abstract

Rat  $\beta_2$ -microglobulin ( $\beta_2m$ ) was isolated from the urine of animals with sodium chromate induced tubular damage. Its physico-chemical properties were similar to those of other  $\beta_2m$  homologues. Rat  $\beta_2m$  displayed immunochemical cross-reactivity with purified  $\beta_2ms$  from other mammals. In rat serum,  $\beta_2m$  was found in high molecular weight complexes and in free form.

Purified rat MHC class 1 antigens (AgB) bound exogenously-added  $\beta_2m$ , both the homologous counterpart or various other mammalian  $\beta_2ms$ , presumably in exchange reactions. Similarly, the  $\beta_2m$  subunit of high molecular weight complexes in mammalian sera apparently could be exchanged for exogenous heterologous  $\beta_2m$ . These data indicate a high degree of conservation of the interacting sites on  $\beta_2m$  and  $\beta_2m$ -binding molecules, respectively. Such interactions can be used in evolutionary studies to detect conserved  $\beta_2m$ -determinants. Also, fish serum contained glycoproteins capable of reacting with a highly conserved part of different mammalian  $\beta_2ms$ .

Heterogeneity of AgB antigen- $^{125}I$ -human  $\beta_2m$  complexes was indicated by immunoprecipitation with different anti-AgB antisera. This suggests that inbred rats produce more than one type of class 1 alloantigen.

Antibodies against guinea pig  $\beta_2m$  reduced the proliferative response of guinea pig lymphocytes to stimulation with mitogens or soluble antigens. This generalizes similar results previously found in the human system.

Anti- $\beta_2m$  antibodies strongly inhibited human NK-activity. The antibodies appeared to operate at the level of the target cells, and interfered selectively with the lytic phase. It was suggested that  $\beta_2m$  may be involved as a target cell acceptor for the lytic message from NK cells.

Key words

$\beta_2$ -microglobulin, protein purification, MHC class 1 antigens, evolution,  $\beta_2$ -microglobulin-binding molecules, fish serum, lymphocyte transformation, NK cells, anti- $\beta_2$ -microglobulin antibodies, immunoglobulin-like domain.

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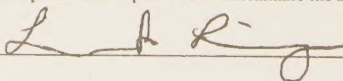
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Lennart Lögdborg



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*A wise old owl lived in an oak;  
The more he saw the less he spoke;  
The less he spoke the more he heard;  
Why can't we all be like that bird?*

*(Unknown)*

## CONTENTS

|                                                                                                                          |    |
|--------------------------------------------------------------------------------------------------------------------------|----|
| LIST OF PAPERS.....                                                                                                      | 5  |
| ABBREVIATIONS.....                                                                                                       | 6  |
| PREVIOUS INVESTIGATIONS.....                                                                                             | 7  |
| Introduction.....                                                                                                        | 7  |
| Metabolism: Synthesis, cell surface expression,<br>presence in biological fluids, catabolism.....                        | 7  |
| Clinical importance of $\beta_2$ -microglobulin.....                                                                     | 11 |
| $\beta_2$ -microglobulin is physically associated with<br>other proteins.....                                            | 11 |
| $\beta_2$ -microglobulin - a member of a protein superfamily.....                                                        | 15 |
| Biological effects of $\beta_2$ -microglobulin and of<br>antibodies directed against this protein.....                   | 16 |
| Summary.....                                                                                                             | 18 |
| THE PRESENT INVESTIGATION.....                                                                                           | 19 |
| Isolation and purification of a rat $\beta_2$ -microglobulin<br>homologue.....                                           | 19 |
| On the evolution of $\beta_2$ -microglobulin, $\beta_2$ -microglobulin-<br>binding molecules, and their interaction..... | 23 |
| Additional class 1 alloantigens in the rat.....                                                                          | 25 |
| Effects of antibodies against $\beta_2$ -microglobulin on<br>lymphocyte transformation in animal models.....             | 26 |
| Effect of antibodies against $\beta_2$ -microglobulin on natural<br>killer cell-mediated cytotoxicity.....               | 27 |
| SUMMARY.....                                                                                                             | 30 |
| ACKNOWLEDGEMENTS.....                                                                                                    | 31 |
| REFERENCES.....                                                                                                          | 32 |
| APPENDIX: PAPERS I - VII.....                                                                                            | 47 |



## LIST OF PAPERS

This thesis is based on the following papers referred to by Roman numerals in the text.

- I. Lögdberg, L., Östergren, P.-O., and Berggård, I. Rat  $\beta_2$ -microglobulin. Isolation, properties and relation to  $\beta_2$ -microglobulins from other species. Molec. Immunol. 16, 577-587 (1979).
- II. Lögdberg, L., Björck, L., and Cigén, R. Heterologous interaction between  $\beta_2$ -microglobulin and AgB-antigens. Transplantation 30, 233-235 (1980).
- III. Lögdberg, L., and Björck, L. On the interaction between  $\beta_2$ -microglobulin and the heavy chain of MHC class 1 antigens. Submitted for publication.
- IV. Lögdberg, L., Cigén, R., and Björck, L. Binding of  $\beta_2$ -microglobulin by heterologous sera. Scand. J. Immunol. 13, 237-243 (1981).
- V. Lögdberg, L., and Björck, L. Binding of mammalian  $\beta_2$ -microglobulin by glycoproteins in fish serum. Submitted for publication.
- VI. Lögdberg, L., Cigén, R., and Berggård, I. Effects of anti-guinea-pig  $\beta_2$ -microglobulin antibodies on lymphocyte transformation induced by specific antigens or mitogens. Scand. J. Immunol. 9, 263-271 (1979).
- VII. Kalland, T., and Lögdberg, L.  $\beta_2$ -Microglobulin - acceptor for the lytic signal from natural killer cells? Submitted for publication.

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## ABBREVIATIONS

|            |                                                 |
|------------|-------------------------------------------------|
| ADCC       | - Antibody-dependent cell-mediated cytotoxicity |
| ALG        | - Anti-lymphocyte globulin                      |
| $\beta_2m$ | - $\beta_2$ -microglobulin                      |
| ConA       | - Concanavalin A                                |
| CTL        | - Cytotoxic T-lymphocytes                       |
| DxS        | - Dextran sulfate                               |
| LDCC       | - Lectin-dependent cell-mediated cytotoxicity   |
| LMW-PP     | - Low molecular weight plasma protein           |
| LPS        | - Lipopolysaccharide                            |
| LT         | - Lymphotoxin                                   |
| MHC        | - Major histocompatibility complex              |
| AgB        | - MHC of rat                                    |
| HLA        | - MHC of man                                    |
| H-2        | - MHC of mouse                                  |
| NK cells   | - Natural killer cells                          |
| PHA        | - Phytohaemagglutinin                           |
| PPD        | - Purified protein derivative                   |

## PREVIOUS INVESTIGATIONS

### Introduction.

The important role of the kidney in the metabolism of low molecular weight plasma proteins (LMW-PP) was delineated during the fifties and sixties (cf. Strober & Waldman, 1974). LMW-PP are to a large extent filtered through the glomerular membrane. Reabsorption occurs when the primary urine passes the proximal tubulus. Thus, the LMW-PP end up in the lysosomes of the proximal tubulus cells, where they are catabolized. In accordance, urine from patients with defects in the proximal tubulus, contains increased amounts of LMW-PP. Therefore, such urine is an excellent source for the purification of LMW-PP. Ingemar Berggård, who had been working with the identification of plasma proteins in normal urine, realized this potential of the pathological urine. Thus, from the urine of patients with tubular proteinuria due to Wilson's disease, or cadmium-poisoning, he was able to isolate several, previously unknown LMW-PP. Among these was a small ( $M_w \approx 12,000$ ), compact, globular polypeptide -  $\beta_2$ -microglobulin ( $\beta_2m$ ) - which will be the subject of the present thesis.  $\beta_2m$  was first mentioned in 1964 (Berggård, 1964), and the purification and physicochemical characteristics of the protein were reported in 1968 (Berggård & Bearn, 1968). This opened up a field for intense investigation, first restricted to the use of  $\beta_2m$  as a model molecule in studies of the renal handling of proteins, but  $\beta_2m$  soon attracted the interest of immunologists. Recent years have also seen the growth of a by now extensive literature on the usefulness of determining  $\beta_2m$ -concentrations in serum, or other body fluids, as a clinical tool during renal, inflammatory, or neoplastic disorders. Table 1 gives an overview of some major findings in the field of  $\beta_2m$ .

### Metabolism: Synthesis, cell surface expression, presence in biological fluids, catabolism.

$\beta_2m$  is structurally related to immunoglobulins (see below). Thus, lymphocytes were the first cell type investigated as the potential cellular origin of  $\beta_2m$ . In this respect, lymphocytes are still the most extensively studied.  $\beta_2m$  is synthesized by lymphocytes (Bernier & Fanger, 1972; Poulik & Bloom, 1973) and is expressed on the cell surface (Peterson et al., 1972), both on T- and B-lymphocytes (Poulik, 1973), in the order  $10^5$  -  $10^6$  molecules per cell (Björck et al., 1979; Dorval et al., 1977; Evrin & Pertoft, 1973; Plesner, 1976).  $\beta_2m$ -synthesis does not correlate well with immunoglobulin-synthesis, indicating separate genetic regulation for these proteins (Hütteroth et al., 1973; Nilsson et al., 1973; Poulik & Bloom, 1973). Also, other cells or cell lines of various histogenetic origin seem to be able to synthesize  $\beta_2m$ , to express it on the cell surface, and to release it to the medium (Evrin & Nilsson, 1974; Nilsson et al., 1973; Nilsson et al., 1974). Thus, it is now believed that most adult somatic cells can produce  $\beta_2m$ . Exceptions to this rule are some  $\beta_2m$ -deficient cell lines (Nilsson et al., 1974; Pious et al., 1976) and mature erythrocytes (Evrin & Pertoft, 1973). Although limited, existing data on *in situ*-expression of  $\beta_2m$  in tissues indicate large differences in  $\beta_2m$ -density on different cell types (Bhan et al., 1980; Chung-Park et al., 1980; Forsum & Malmnäs-Tjernlund, 1977; Governa & Biguzzi, 1976; Ooi

**Table 1.** A short history of  $\beta_2$ -microglobulin.

| Year    | Finding                                                                                                                               | References                                                                                                     |
|---------|---------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|
| 1964    | First mentioned in the scientific literature                                                                                          | Berggård, 1964.                                                                                                |
| 1968    | Purification schedule and physico-chemical characterization                                                                           | Berggård & Bearn, 1968.                                                                                        |
| 1968-   | Kidney identified as the site of catabolism. Model protein for studies of renal function.                                             | Bernier et al., 1968; Bernier & Conrad, 1969; Peterson et al., 1969; Wibell et al., 1973; and several others.  |
| 1972-   | Purification of $\beta_2$ m-homologues from various species, including dog, rabbit, mouse, guinea pig, rat, cow, chicken, and turkey. | see Table 4.                                                                                                   |
| 1972-74 | Demonstration of synthesis, cell surface expression, and secretion of $\beta_2$ m by most cell types.                                 | Bernier & Fanger, 1972; Peterson et al., 1972; Nilsson et al., 1973, 1974; Poulik, 1973; Poulik & Bloom, 1973. |
| 1972    | Determination of amino acid sequence. Homology to immunoglobulin domains.                                                             | Peterson et al., 1972; Smithies & Poulik, 1972 b.                                                              |
| 1973    | First demonstration of elevated plasma levels of $\beta_2$ m in patients with inflammatory or neoplastic diseases.                    | Evrin & Wibell, 1973.                                                                                          |
| 1973    | Interaction of anti- $\beta_2$ m with lymphocyte transformation. This inspired the studies summarized in Table 2.                     | Bach et al., 1973.                                                                                             |
| 1973-74 | $\beta_2$ m was shown to be physically associated with human MHC class I antigen heavy chain.                                         | Grey et al., 1973; Nakamuro et al., 1973; Peterson et al., 1974.                                               |

**Table 1** Continued.

| Year  | Finding                                                                                                                                                                                          | References                                                                                                                                                                                                                                             |
|-------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1975  | The human $\beta_2m$ gene located to chromosome 15.                                                                                                                                              | Goodfellow et al., 1975.                                                                                                                                                                                                                               |
| 1975- | Association detected between $\beta_2m$ and molecules other than classical MHC class 1 antigens. Include the Qa1, Qa2,3, and TL alloantigens, serum components, H-Y antigen, and tumor antigens. | Callahan & Allison, 1978;<br>Fellous et al., 1978;<br>Kvist & Peterson, 1978;<br>Michaelson et al., 1977;<br>Natori et al., 1976;<br>Östberg et al., 1975;<br>Stanton & Hood, 1975;<br>Thomson et al. 1976;<br>Vitetta et., 1975 b and several others. |
| 1978- | Expansion of the class of proteins containing immunoglobulin/ $\beta_2m$ -like domains to include the MHC class 1 antigen heavy chain, the MHC class 2 antigen chains, and Thy-1 antigen.        | Campbell et al., 1979;<br>Kaufman & Strominger, 1982;<br>Kratzin et al., 1981;<br>Larhammar et al., 1981;<br>Lee et al., 1982;<br>Orr et al., 1979;<br>Trägårdh et al., 1979.                                                                          |
| 1979  | Cell-free synthesis of $\beta_2m$ .                                                                                                                                                              | Lingappa et al., 1979;<br>Ploegh et al., 1979.                                                                                                                                                                                                         |
| 1980  | First example of $\beta_2m$ -allelism (mouse). This is used to locate the mouse $\beta_2m$ gene to chromosome 2.                                                                                 | Cox et al., 1982;<br>Gates et al., 1981;<br>Goding, 1981; Goding & Walker, 1980;<br>Michaelson, 1981;<br>Michaelson et al., 1980;<br>Robinson et al., 1981, 1982.                                                                                      |
| 1981  | Isolation of cDNA for human and mouse $\beta_2m$ .                                                                                                                                               | Parnes et al., 1981;<br>Suggs et al., 1981.                                                                                                                                                                                                            |
| 1982  | Structure of the mouse $\beta_2m$ gene.                                                                                                                                                          | Parnes & Seidman, 1982.                                                                                                                                                                                                                                |



et al., 1977). In fact, in the most extensive study (Governa & Biguzzi, 1976), several cell types were not stained with anti- $\beta_2m$  in indirect immunofluorescence. The negative results do not necessarily mean that such cells do not express  $\beta_2m$ . Rather, the cells could carry  $\beta_2m$  in a density below the detection limit of the technique, or, alternatively, artefacts could be introduced during the preparation of tissue sections.

In spite of speculations on the role of  $\beta_2m$  and  $\beta_2m$ -associated molecules during embryonal development (Ohno, 1977), little is known about the embryonal expression of  $\beta_2m$ . Embryonal carcinoma (primitive teratocarcinoma) cells, used as models for morula cells (cf. Jacob, 1977), apparently do not express  $\beta_2m$  (Croce et al., 1981; Dubois et al., 1976). When these cells are allowed to differentiate *in vitro*, a 100-fold increase in the transcription of the  $\beta_2m$ -gene is observed (Morello et al., 1982). On the other hand, expression of both the maternal and paternal  $\beta_2m$ -genes were recently demonstrated in embryos, even at the two-cell stage (Sawicki et al., 1981; see also Håkansson & Peterson, 1976).

$\beta_2m$  and the associated transplantation antigens (see below) were reported to be absent from trophoblasts (Faulk & Temple, 1976). This finding could help to explain how the growing fetus avoids being rejected as an allograft. Though this is supported by the absence of  $\beta_2m$  in gestational choriocarcinoma cell lines (Tanaka et al., 1981), more recent studies on the expression of transplantation antigens on the placenta trophoblasts give a more complex picture (cf. Searle, 1982). The presence of  $\beta_2m$  and transplantation antigens on spermatozoa is also a matter of controversy, but  $\beta_2m$  appears to be expressed at least during early stages of spermatogenesis (Anderson et al., 1982; Dubois et al., 1976; Fellous et al., 1976; Law & Bodmer, 1978; Söderström et al., 1982).

From mouse data, it appears that  $\beta_2m$  is encoded by a single gene per haploid genome (Parnes & Seidman, 1982). In man, this gene is situated on chromosome 15 (Faber et al., 1976; Goodfellow et al., 1975; Jones et al., 1976; Smith et al., 1976). Fig. 2 demonstrates the structure of the  $\beta_2m$ -gene. Cell-free translation of this gene reveals that newly synthesized  $\beta_2m$  contain an N-terminal signal peptide of 20 amino acid residues (Lingappa et al., 1979; Ploegh et al., 1979), encoded by the first exon of the  $\beta_2m$ -gene (Parnes and Seidman, 1982). During protein maturation in the ER-Golgi-apparatus, this peptide is removed.

The newly synthesized  $\beta_2m$  is apparently released to the surrounding medium both by secretion and by shedding during cell membrane turnover (cf. Nilsson et al., 1974). Accordingly,  $\beta_2m$  has been detected in all normal biological fluids examined (Berggård & Bearn, 1968; Čejka et al., 1974; Colle et al., 1976; Evrin et al., 1971; Hall & Roux, 1974; Jonasson et al., 1974; Talal et al., 1975).

Turnover studies indicate that humans synthesize about 0.13 mg  $\beta_2m$  per hour and kg (Karlsson et al., 1980 a). Almost all of this is catabolized in the kidney by the proximal tubulus cells. This is demonstrated by elevated serum levels of  $\beta_2m$  in patients with decreased glomerular filtration rate (Bernier et al., 1968; Peterson et al., 1969), and by dramatically increased excretion of  $\beta_2m$  in the urine of patients with defects in the proximal tubulus (Peterson et al., 1969). In fact, the serum concentration of  $\beta_2m$  is better correlated to the glomerular filtration rate than is serum creatinine (Wibell et al., 1973). Similarly, the urinary output of  $\beta_2m$  is a potentially valuable marker for monitoring of the proximal tubular function (cf. Cabrera et al., 1982; Hansen et al.,



1981; Stewart & Hughes, 1981). The catabolic role of the kidney is also demonstrated by studies on the elimination of exogenously administered  $\beta_2m$  in the rat (Bernier & Conrad, 1969; Conway & Poulik, 1977; Maunsbach & Christensen, 1974), and by a wealth of additional studies on patients (cf. review articles indicated below).

### Clinical importance of $\beta_2$ -microglobulin.

Clinical studies constitute a large proportion of the literature in the field of  $\beta_2m$  research. It is beyond the scope of this thesis to treat this subject in depth. Instead, the reader is referred to recent review articles or symposia (Karlsson et al., 1980 b; Pathologie Biologie, 1978; Pesce et al., 1981; Poulik, 1980; Poulik et al., 1979; Uthmann & Geisen, 1981).

To summarize, apart from the use of  $\beta_2m$  in studies of renal function as related in the previous chapter, raised levels of  $\beta_2m$  in various biological fluids have been found in patients suffering from inflammatory or neoplastic diseases. Among such inflammatory diseases are rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's disease, and Crohn's disease. A large number of different neoplastic conditions have been reported to be associated with elevated plasma levels of  $\beta_2m$ , notably various lymphoproliferative disorders. Most authors have corrected for the glomerular filtration rate, so the findings do not simply reflect renal insufficiency secondary to the diseases. In both groups of diseases, the high  $\beta_2m$  levels could be due to increased production by an activated immune system. However, there are studies supporting the possibility that tumor production itself is responsible for the elevated plasma  $\beta_2m$ .

Another intriguing finding is the presence of auto-antibodies against  $\beta_2m$  in patients with RA and SLE.

### $\beta_2$ -microglobulin is physically associated with other proteins.

$\beta_2m$  is water-soluble and it probably does not exist on the cell surface in free form. Thus, one would expect that  $\beta_2m$  binds to the cell surface through interaction with integral membrane proteins. Indeed, this was found to be the case. Human  $\beta_2m$  forms a non-covalently bound complex with the MHC (major histocompatibility complex) class 1 antigens (HLA-A, B, and C), both in solution (Grey et al., 1973; Nakamuro et al., 1973; Peterson et al., 1974) and on the cell surface (Neuport-Sautes et al., 1974; Östberg et al., 1974; Poulik et al., 1973; Solheim & Thorsby, 1974).

The MHC was originally discovered as an immunological barrier against transplanted foreign tissues. Thus, an allografted organ is usually rejected, unless the recipient is kept under immunosuppression. The major reason for the histoincompatibility is the difference between donor and recipient at a specific genetic locus. This was first demonstrated in mice (Gorer et al., 1937), but is also true in man (Dausset et al., 1965; van Rood et al., 1965). The mouse MHC is located on chromosome 17 and is called the H-2 locus, whereas the human MHC was named HLA and is situated on chromosome 6. Similar loci exist in a number of other animal species (cf. Götze, 1977).

Centromere

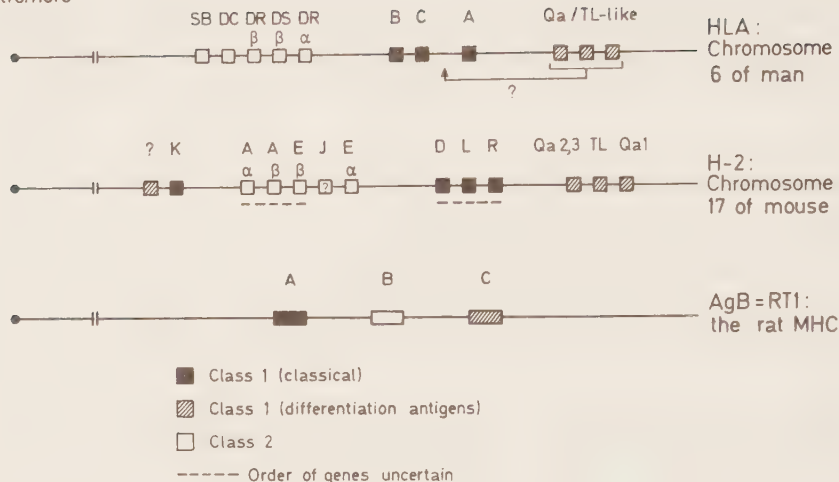
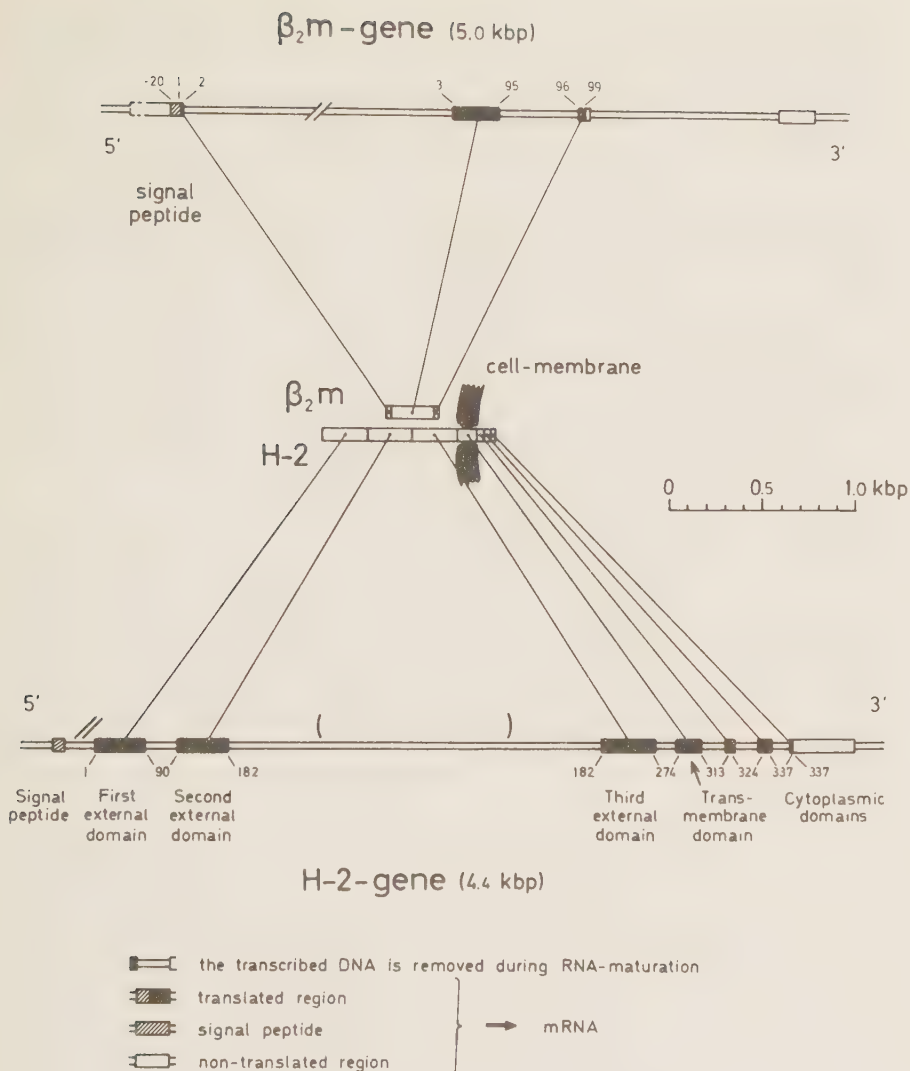


Figure 1.

Gene order in the human, mouse, and rat major histocompatibility complexes. The figure is modified from Robertsson (1982) and Günther & Stark (1979). Genes encoding MHC class 3 antigens (complement factors) were not included (cf. Klein, 1981).

Genes at the MHC encode at least two groups of serologically detectable, highly polymorphic cell membrane proteins, the class 1 and class 2 antigens (cf. Klein, 1979, 1981). Fig. 1 demonstrates the sublocus distribution of the genes for these antigens. The mature product of a class 1 gene is a glycoprotein, consisting of a single polypeptide chain of approximately 340 amino acid residues (cf. Coligan et al., 1981). The extracellular portion of the protein carries the alloantigenicity, is non-covalently associated with  $\beta_2m$ , and is folded into three domains of equal size (Fig. 2). A hydrophobic part of the molecule - the transmembrane domain - is responsible for the membrane integration, leaving a short hydrophilic segment of the molecule at the cytoplasmic side of the membrane. This subdivision of the molecule is also seen at the DNA level (Fig. 2) (Malissen et al., 1982; Moore et al., 1982; Steinmetz et al., 1981 a,b). The association with  $\beta_2m$  is apparently necessary for normal expression of the alloantigenicity (Lancet et al., 1979; Vincent & Revillard, 1979), and  $\beta_2m$  is thought to stabilize the conformation of the class 1 antigen heavy chain.  $\beta_2m$ -association is probably also important for the normal post-translational modification and transport of the heavy chain (cf. Segre et al., 1981).

Class 2 antigens consist of two non-covalently associated, membrane-integrated glycoproteins (cf. Robertsson, 1982). Both polypeptide chains have an approximate length of 230 amino acid residues. Most of the observed polymorphism seems to reside in the  $\beta$ -chain. The extracellular portion of both chains fold into two domains, and, like the class 1 antigen heavy chain, both have a hydrophobic transmembrane domain and a hydrophilic cytoplasmic domain. Again, the subdivision at the protein level is reflected in a similar subdivision at the DNA level (Lee et al., 1982).



**Figure 2.**

Structure of the genes encoding the two polypeptide chains of the MHC class 1 antigens. The  $\beta_2m$  gene structure is from Parnes & Seidman, (1982). The H-2 class 1 gene structure is from Steinmetz et al. (1981 b) and modified according to the data of Moore et al. (1982).

Because of the high polymorphism of the MHC, two unrelated individuals will usually have different alleles at their corresponding class 1 and class 2 gene subloci. Thus, after transplantation between two such individuals, the immune apparatus of the recipient will recognize as foreign the class 1 and class 2 antigens on the donor tissue. This starts a series of events, which eventually ends with graft rejection.

It does not seem reasonable, however, to believe that evolution has created a system as ubiquitous and complex as the MHC, and maintained it in such an astounding state of polymorphism, just to enable the individual immune system to recognize foreign tissues. In vertebrates, such a system could normally be useful only in experimental situations. Instead, many findings, including the linkage between MHC-alleles and diseases (cf. Möller, 1975), point to a central role for the MHC in normal immune reactions. The MHC antigens appear to be restricting elements in T-lymphocyte recognition (cf. Benacerraf, 1978; Cohn & Epstein, 1978; Doherty et al., 1976; Rosenthal, 1978; Shearer & Schmitt-Verhulst, 1977). Thus, the cytotoxic T-lymphocytes (CTL) recognize antigen in the context of self MHC class 1 antigens. Similarly, class 2 antigens restrict the interaction between antigen-presenting macrophages and helper T-lymphocytes, as well as the interaction between helper T-lymphocytes and B-lymphocytes. In this way, the MHC can influence the induction, regulation, and effectuation of an immune response.

$\beta_2m$  is a part of the MHC class 1 antigens in all species investigated. It is also structurally related to the class 2 antigens (see below). In addition, other cell surface alloantigens, encoded by genes linked to the MHC on mouse chromosome 17, are physically associated with  $\beta_2m$  (Michaelson et al., 1977, 1981; Östberg et al., 1975; Rothenberg & Trigila, 1981; Stanton & Hood, 1980; Vitetta et al., 1975 b; Yokoyama et al., 1982). These molecules are the Qa1, Qa2,3, and TL alloantigens. They are structurally related to the classical MHC class 1 antigens, both at the protein level (McIntyre et al., 1982; Pischel & Little, 1980; Soloski et al., 1981, 1982 a,b; Yokoyama et al., 1981) and the DNA level (Margulies et al., 1982; Steinmetz et al., 1981b, 1982). Thus, one can speculate that chromosome 17 harbours a superfamily of class 1 genes, which arose during evolution through duplications and recombinations of a common ancestral class 1 gene (cf. Kindt, 1981). Interestingly, with DNA-probes one finds more class 1 genes than can presently be accounted for by serological data (Cami et al., 1981; Margulies et al., 1982; Pease et al., 1982; Steinmetz et al., 1982). Steinmetz et al. (1982) reported 36 genes, divided into 13 clusters. This should be compared to the 5-10 class 1 subloci that have been distinguished serologically (Fig. 1). At least one class 1 gene maps centromeric to the H-2 K locus (Pease et al., 1982). Some of all these DNA-sequences may represent pseudogenes. Others may encode known serological activities, like the Qa4 and Qa5 antigens (Hämmerling et al., 1979), or hitherto unidentified molecules.

It should be noted that the Qa and TL antigens differ from classical class 1 antigens in some respects. They are only modestly polymorphic, and they have a restricted tissue distribution, being mainly present on various lymphocyte subpopulations (cf. Lynes et al., 1982; Nell et al., 1982; Yokoyama et al., 1981). Thus, they are thought to be lymphocyte differentiation antigens. Possible Qa and TL homologues have been identified in man (cf. Gazit et al., 1980; Knowles & Bodmer, 1982), guinea pig (Schwartz et al., 1978), and rat (Haustein et al., 1982; Stock & Günther, 1982).

$\beta_2m$  or  $\beta_2m$ -determinants are apparently also a part of a number of additional molecules. These include a serum protein, lacking in conventional alloantigenic activity, but with a class 1-like structure (Kvist & Peterson, 1978; Natori et al., 1976; Ramanathan et al., 1982). The genetic control of this protein is linked to the MHC (Kvist et al., 1980 b), and it may be a product of an unusual class 1 gene. This gene (Cosman et al., 1982) differs from other class 1 genes, in that it cannot encode the transmembrane domain. Its product is therefore expected to be secreted. Expression of this gene has only been detected in the liver, where it represents about 25 % of all class 1-specific transcripts. Other groups report presence of  $\beta_2m$ -determinants on other serum molecules (Callahan et al., 1976; Katz, 1978), on cell-cell interaction factors (Armerding et al., 1975; Lamb et al., 1981), and on the cell surface-expressed H-Y antigen (Fellous et al, 1978; Müller et al., 1979). The latter antigen has been implicated as the prime inducer of heterogametic sex (cf. Wachtel, 1977). Its association with  $\beta_2m$  stimulated the hypothesis that  $\beta_2m$  may be involved in embryonal differentiation. An exciting preliminary report that  $\beta_2m$  is associated with an antigen specific for morula cells (F9) (Vitetta et al., 1975 a) was not verified (Vitetta et al., 1977 a).

One of the mechanistic interpretations of the MHC restriction of CTL - the altered self hypothesis - predicts that antigens physically interact with the self MHC class 1 antigens, thereby altering their conformation. These changed self MHC antigens are then recognized as foreign by the CTL. If this is true, one could expect tumor antigens to be associated with  $\beta_2m$  on the cell surface of the tumor cells. Indeed, such an association was found by several authors (e.g., Callahan et al., 1978; Malley et al., 1979; Thomson et al., 1976). Other investigators have failed to demonstrate this type of interaction (e.g., Bowen & Baldwin, 1979; McCabe et al., 1980). Whether this means that the finding is not valid for all tumors, or if failure to detect the association is a technical artefact, is still an open question.

### $\beta_2$ -microglobulin - a member of a protein superfamily.

Antibodies (immunoglobulins) are built up by several compact, globular structures, the immunoglobulin domains. These units, comprising approximately 100 amino acid residues each, not only share physico-chemical properties, but also show amino acid sequence homology when compared to each other (Edelman et al., 1969). This forms a basis for the hypothesis that immunoglobulin genes evolved by duplications and recombinations of an ancestral gene (Hill et al., 1966; Singer & Doolittle, 1966). Recent, detailed studies on the structure of the immunoglobulin genes strongly support this hypothesis (cf. Gottlieb, 1980; Marx, 1981). The determination of the  $\beta_2m$ -sequence (Cunningham et al., 1973; Peterson et al., 1972; Smithies & Poulik 1972 b) led to the discovery that  $\beta_2m$  is as homologous to the constant immunoglobulin domains as these are to one another. More recently, the third external domain of the MHC class 1 antigen heavy chain was shown to be an immunoglobulin-like domain, by the same criterion (Orr et al., 1979; Trägårdh et al., 1979). This supports the postulate that immunoglobulin genes evolved from genes specifying histocompatibility antigens (Bodmer, 1972; Gally & Edelman, 1972). The structural relation of the two MHC class 1 antigen chains to immunoglobulins also extends to the gene structures of these molecules (Malissen et al., 1982; Moore et al., 1982; Parnes & Seidman, 1982; Steinmetz et



al., 1981 b). In addition, immunological cross-reactions between  $\beta_2m$  and immunoglobulins have been demonstrated (Gottlieb et al., 1977; Vincent & Revillard, 1980).

Class 1 antigens encoded by genes outside the MHC, like the Qa and TL alloantigens, are also expected to contain immunoglobulin-like domains. Recent data on class 1 gene structure support this notion (Steinmetz et al., 1982). The finding that the two MHC class 2 antigen chains also contain immunoglobulin-like domains (Kaufman & Strominger, 1982; Kratzin et al., 1981; Larhammar et al., 1981; Lee et al., 1982), suggests a common evolutionary pathway for all MHC antigens, other class 1 antigens,  $\beta_2m$ , and immunoglobulins.

Thus, in man, at least five different chromosomes contain genes encoding immunoglobulin-like domains: chromosome 2 (immunoglobulin  $\kappa$ -light chains), 6 (MHC), 14 (immunoglobulin heavy chains), 15 ( $\beta_2m$ ), and 22 (immunoglobulin  $\lambda$ -light chains) (Croce et al., 1979; Dausset et al., 1965; Erikson et al., 1981; Goodfellow et al., 1975; McBride et al., 1982; van Rood et al., 1965). The Thy-1 antigens and Thymopoeitin are claimed to be additional members of this protein superfamily (Campbell et al., 1979; Cohen et al., 1981; Hahn & Hamburger, 1981; Williams & Gagnon, 1982).

#### Biological effects of $\beta_2$ -microglobulin and of antibodies directed against this protein.

To obtain clues to the function of  $\beta_2m$ , the protein, or antibodies against it, were added to various biological systems. This chapter reviews some of the observed effects. For a more detailed discussion, see Berggård et al. (1980).

$\beta_2m$  seems to share some functional properties with the domains in the Fc-fragment of immunoglobulins. Thus,  $\beta_2m$  can promote macrophage rosetting of sheep red blood cells, and is also able to fix complement (Painter et al., 1974). In addition,  $\beta_2m$  can enhance expression of Fc-receptors for IgG on the lymphocyte surface (Birch et al., 1979), it may have chemotactic properties (Conway & Poulik, 1976, Fed. Proc. 35, 515, abstract), it may perhaps have anticoagulant activity (Hammerschmidt & Moldow, 1978), and it can serve as a substrate of tissue transglutaminase (Fésüs et al., 1981).

Anti- $\beta_2m$  antibodies have many biological effects, probably exerted at several levels of the immune system (see Tab. 2 for references). Both lymphocyte transformation induced by various stimuli, and the action of various cytotoxic effector cells, can be inhibited by anti- $\beta_2m$ . This implies involvement of  $\beta_2m$  in both the induction and the effector phases of an immune response. Thus, it is perhaps not surprising to find that anti- $\beta_2m$  can prolong graft rejection. These effects will be further discussed below.

All the effects recorded in Table 2 are not necessarily due to functional involvement of  $\beta_2m$ . For instance, F(ab')<sub>2</sub>-fragments of anti- $\beta_2m$  does not inhibit ADCC or EA-rosette formation. Instead, the inhibitory effect of complete anti- $\beta_2m$  antibodies could be caused by co-capping of Fc-receptors on the effector cells, by the Fc-fragments of anti- $\beta_2m$  bound to effector cell surface  $\beta_2m$ . Some of the other effects could simply be due to steric



**Table 2** Biological effects of anti- $\beta_2$ -microglobulin.

| Biological assay                      | Species    | Effect of anti- $\beta_2$ m                            | References |
|---------------------------------------|------------|--------------------------------------------------------|------------|
| Lymphocyte transformation induced by: |            |                                                        | a          |
| Allogeneic cells (MLR)                | Man        | Inhibition                                             |            |
| Soluble antigen (PPD)                 | Man        | Inhibition                                             |            |
| T-lymphocyte mitogens (PHA, ConA)     | Man        | Inhibition                                             |            |
| Antiserum (ALG)                       | Man        | Inhibition                                             |            |
| Spontaneous lymphocyte transformation | Man, Mouse | Enhancement                                            | b          |
| Cell-mediated cytotoxicity:           |            |                                                        | c          |
| CTL-mediated                          | Man        | Inhibition                                             |            |
|                                       | Mouse      | Inhibition                                             |            |
| ADCC                                  | Man        | Inhibition                                             |            |
| LDCC                                  | Man        | Inhibition                                             |            |
| Macrophage-mediated                   | Man        | Inhibition                                             |            |
| Allograft rejection                   | Rat        | Prolongation                                           | d          |
| Rosette tests:                        |            |                                                        | e          |
| E (T-lymphocytes)                     | Man        | None                                                   |            |
| EAC (Cells with complement receptors) | Man        | Variable                                               |            |
| EA (Cells with Fc-receptors)          | Man        | Variable, but several groups report strong inhibition. |            |
| Cell migration:                       |            |                                                        | f          |
| Leukocyte migration                   | Man        | Inhibition                                             |            |
| Monocyte migration                    | Man        | Chemotaxis                                             |            |

<sup>a</sup>Bach et al., 1973, 1976; Leino et al., 1981; Lindblom et al., 1974; McCalmon et al., 1975 a, b, 1980; Östberg et al., 1976; Solheim, 1974; Solheim & Thorsby, 1974; Vincent et al., 1975.

<sup>b</sup>Kin et al., 1979; Möller & Persson, 1974; Östberg et al., 1976; Ringdén, 1979, 1980; Ringdén & Johansson, 1977; Ringdén & Möller, 1975; Ringdén et al., 1978; Solheim, 1974; Solheim & Thorsby, 1974.

<sup>c</sup>Bach et al., 1976; McMichael et al., 1980; Saxena et al., 1982; Solheim et al., 1976; Tomonari et al., 1982; Vincent et al., 1975.

<sup>d</sup>Björck et al., 1977 b.

<sup>e</sup>Galili et al., 1977; Grey et al., 1975; Morito et al., 1978; Sarmay et al., 1979, 1980; Solheim et al., 1976; Vincent et al., 1975.

<sup>f</sup>Conway & Poulik, 1981; Lögdberg, unpublished.

blockade of unrelated cell surface antigens. However, many of the effects could be seen also with Fab'- and F(ab')<sub>2</sub>-fragments. The use of monoclonal antibodies against  $\beta_2m$  may also help to define more clearly which of the effects that are really related to the function(-s) of  $\beta_2m$ .

#### Summary.

In summary,  $\beta_2m$  is a LMW protein, originally isolated from urine, that can be synthesized by most cell types, released into the surrounding medium, and eliminated by the kidney.  $\beta_2m$ -producing cells also usually express the protein on their cell surface, in physical association with MHC class 1 antigen heavy chains or other related molecules.  $\beta_2m$  enter a protein superfamily together with immunoglobulins, both classes of MHC antigens, and some additional molecules. The members of this protein family probably share a common evolutionary origin. Despite the numerous biological effects recorded for  $\beta_2m$  and anti- $\beta_2m$ , the functional role of  $\beta_2m$  is uncertain.

## THE PRESENT INVESTIGATION

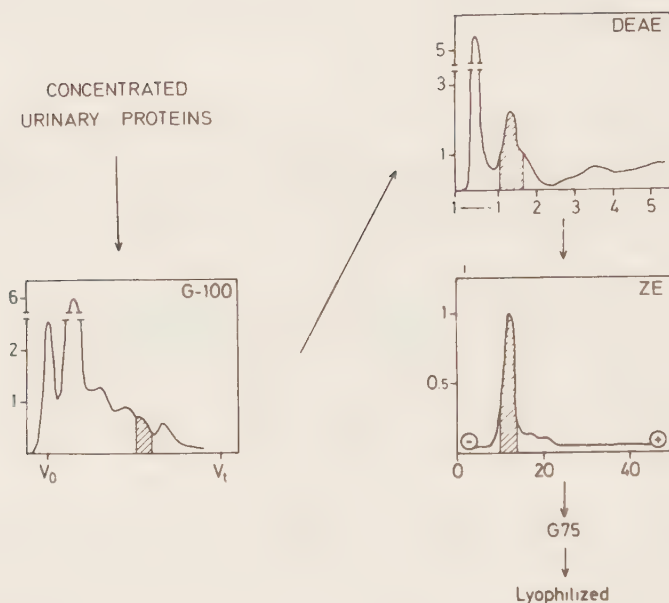
### Isolation and purification of a rat $\beta_2$ -microglobulin homologue (I).

To be able to investigate the role of  $\beta_2$ m in more detail, it is necessary to work with animal models. A prerequisite for such studies is the purification of animal  $\beta_2$ m-homologues, and to use the purified protein to prepare specific antisera.

The rat is one of the most widely studied laboratory animal species, with an availability of inbred and congenic strains. For preparative work, the rat is superior to the otherwise preferable mouse model. Therefore, we decided to purify the putative rat  $\beta_2$ m-homologue. Based on previous experience (Berggård, 1974; Evan & Dail Jr, 1974), we used sodium chromate to induce proximal tubular damage in the rat kidneys. The rats reacted to sodium chromate injections with increased daily  $\beta_2$ m-excretion, reaching a maximum (~400-fold increment) on day 3.

Rat  $\beta_2$ m was purified from the urine by gel chromatography on Sephadex G-100, followed by DEAE-cellulose ion-exchange chromatography, zone electrophoresis in Pevicon blocks, and, finally, repeated gel chromatography on Sephadex G-75 or G-100 (Fig. 3). This is similar to the strategy we presently use for purification of human, rabbit, or guinea pig  $\beta_2$ ms (cf. Berggård et al., 1980). Rat  $\beta_2$ m has also been isolated by Poulik (cf. Poulik & Smithies, 1979), and recently by Furuhamu & Onodera (1982). Poulik used only gel filtration followed by ion-exchange chromatography on DEAE-cellulose. Furuhamu & Onodera used ammonium sulfate precipitation, gel chromatography, ion-exchange chromatography, zone electrophoresis, and isoelectric focusing.

The purified rat  $\beta_2$ m migrates as a sharp band in the  $\gamma$ -region on agarose electrophoresis at pH 8.6. It contains small amounts of a more acidic component, which appeared to be another form of rat  $\beta_2$ m. This component could be removed by an alternate scheme of purification, employing isoelectric focusing instead of zone electrophoresis as a third purification step. Interestingly, two or more electrophoretically different forms of urinary  $\beta_2$ m were also detected in man (Hall et al., 1977; Poulik, 1975; Tanahashi et al., 1979 a), guinea pig (Cigén et al., 1978), and rabbit (Björck & Berggård, 1981). The more acidic forms have often been dismissed as due to urinary deamidation. The finding of multiple electrophoretical forms also in serum (Ragnhild Cigén, personal communication) and colostrum (Tanahashi et al., 1979 b) indicate instead that they could be functional charge-isomers. Whether the multiple forms represent genetical variation or post-translational modifications, respectively, remains to be established. Allelism, due to amino acid substitution in position 85 of the sequence, was recently demonstrated in the mouse by several groups (Gates et al., 1981; Goding & Walker, 1980; Michaelson et al., 1980; Robinson et al., 1981 a).



**Figure 3**

Purification of rat  $\beta_2m$ -microglobulin.

The figure is modified from Berggård et al. (1980). The graphs show protein distribution (absorbance at 280 nm) plotted against elution volume (G-100), conductivity ( $m\Omega^{-1} cm^{-1}$ ; DEAE), or fraction number (ZE).

Some physico-chemical properties of rat  $\beta_2m$  are compared to those of human  $\beta_2m$  in Table 3. In collaboration with Sundelin et al. (unpublished), we have determined the amino acid sequence of rat  $\beta_2m$ . Fig. 4 presents this sequence in comparison with the sequences of other  $\beta_2m$ -homologues. The Figure demonstrates 61 - 86 % sequence homology between various  $\beta_2m$ s. The rat  $\beta_2m$ -sequence matches almost perfectly the reported amino acid composition. The only difference is an extra glutamic acid-residue in the composition, which was calculated on the basis of 100 residues, in contrast to the sequence of 99 residues found.

We measured a serum level of about 5  $\mu g$  rat  $\beta_2m$  per ml, which was confirmed by Furuham & Onodera (1982). Part of the  $\beta_2m$  in rat serum exists as high molecular weight complexes, presumably containing  $\beta_2m$  bound to class 1 antigen-like molecules (see discussion in the review chapter). Even in urine, a small fraction of the  $\beta_2m$  (~1%) was found in a high molecular weight complex, with a size similar to that in serum.





**Table 3** Some physico-chemical properties of rat and human  $\beta_2$ -microglobulins.

|                                                              | Rat $\beta_2m^a$    | Human $\beta_2m^b$ |
|--------------------------------------------------------------|---------------------|--------------------|
| Sedimentation coefficient, $S_{20, w}$ (S)                   | 1.6 <sup>c</sup>    | 1.6                |
| Stokes' molecular radius, $r_s$ (nm)                         | 1.6 <sup>c</sup>    | 1.6                |
| Apparent diffusion coefficient, $D_{20, w}$ ( $\mu m^2/s$ )  | 130 <sup>c</sup>    | 133                |
| Partial specific volume, $v$ (ml/g)                          |                     |                    |
| Calculated from amino acid composition                       | 0.734               | 0.727              |
| Frictional ratio, $f/f_0$                                    | 1.13 <sup>c</sup>   | 1.16               |
| Molecular weight                                             |                     |                    |
| - From sedimentation and diffusion constants                 | 12,400 <sup>c</sup> | 11,600             |
| - By sedimentation equilibrium ultracentrifugation           | 11,700              |                    |
| - By SDS-polyacrylamid gel electrophoresis <sup>d</sup>      | 11,800              |                    |
| - By gel chromatography in 6M Gdn-HCl <sup>d</sup>           | 12,300              |                    |
| - From amino acid composition                                | 11,750              | 11,815             |
| Absorption coefficient at 280 nm, pH 7.0 ( $M^{-1}cm^{-1}$ ) |                     |                    |
| Found                                                        | $1.42 \times 10^4$  | $1.98 \times 10^4$ |
| Calculated                                                   | $1.62 \times 10^4$  | $1.94 \times 10^4$ |
| Isoelectric point (electrophoresis)                          | 7.2                 | 5.8                |
| Free sulphydryl groups                                       | nil <sup>e</sup>    | nil                |

<sup>a</sup>Data from paper I except when otherwise indicated.

<sup>b</sup>Data from Berggård & Bearn, 1968 and Karlsson, 1974.

<sup>c</sup>Data from Kvist et al. 1980 a.

<sup>d</sup>Human  $\beta_2m$  was used as reference protein.

<sup>e</sup>Own unpublished data.

Rat  $\beta_2m$  and antibodies directed against it, produced by us or others, have turned out to be useful reagents. We used them in studies presented in this thesis or elsewhere (e.g., Björck et al., 1977 b, 1979). They have also facilitated work in the rat model by others investigators, e.g., in studies on the catabolism of  $\beta_2m$  (Conway & Poulik, 1977), for monitoring during purification of rat transplantation antigens (Kvist et al., 1980 a; see also below), and in the studies of physical interactions between viral and MHC antigens (Kvist et al., 1978 b). Rat and mouse  $\beta_2m$  are closely related (cf. Fig. 4), and the expectation that antibodies against rat  $\beta_2m$  could be useful for studies in the mouse model has been fulfilled (Callahan et al., 1976; Geib et al., 1976; Michaelson et al., 1977; Rothenberg & Triglia, 1981; Stanton & Hood, 1980; Vitetta et al., 1976; etc.).

On the evolution of  $\beta_2$ -microglobulin,  $\beta_2$ -microglobulin-binding molecules, and their interaction (I-V).

The membership of  $\beta_2$ m and  $\beta_2$ m-binding molecules in a protein superfamily (see above), motivate studies on the evolution of these molecules. It is evident from Fig. 4 that the  $\beta_2$ m-sequence has been conserved during mammalian speciation. Some parts of the sequence show particularly little variation between species, e.g., around the cysteins that are involved in the intra-chain disulfide loop. The conservation of the primary structure is also reflected in similar physico-chemical properties, and mammalian  $\beta_2$ m-homologues show extensive immunological cross-reactions with each other (cf. paper I).

**Table 4.** Purification of  $\beta_2$ -microglobulin homologues.

| Animal species | Source                                                    | References                                                                                        |
|----------------|-----------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| Human          | Urine<br>Milk-Colostrum<br>Lymphocyte culture supernatant | Berggård & Bearn, 1968;<br>Čejka et al., 1976;<br>Nakamuro et al., 1973;<br>Poulik & Bloom, 1973. |
| Dog            | Urine                                                     | Smithies & Poulik, 1972 a.                                                                        |
| Rabbit         | Urine                                                     | Berggård, 1974.                                                                                   |
| Mouse          | Urine<br>Liver cell membranes                             | Graf et al., 1982;<br>Natori et al., 1975.                                                        |
| Cow            | Milk-Colostrum                                            | Groves & Greenberg, 1977.                                                                         |
| Guinea pig     | Urine                                                     | Cigén et al., 1978.                                                                               |
| Rat            | Urine                                                     | Paper I; cf. Poulik & Smithies, 1979.                                                             |
| Chicken        | Serum                                                     | Winkler & Sanders, 1977.                                                                          |
| Turkey         | Serum                                                     | Lillehoj et al., 1982.                                                                            |

The only non-mammalian species from which  $\beta_2$ m-homologues have been isolated are some birds (see Table 4). Several studies indicate that the chicken  $\beta_2$ m is a part of MHC class I antigen-homologues on the surface of chicken lymphocytes (Kvist et al., 1978 a; Vitetta et al., 1977 b). Strong biochemical evidence for the presence of MHC/ $\beta_2$ m-homologues in other non-mammalian animal classes has not been reported. However, the widespread finding of strong histoincompatibility reactions, even in invertebrates (cf. Cooper, 1981), suggests the presence of an MHC, at least throughout the vertebrate line.

The very reason for antibody-production is that the  $\beta_2$ m used for immunization differs from the  $\beta_2$ m of the immunized animal. As a consequence, most antibodies produced against  $\beta_2$ m probably do not recognize conserved determinants on the molecule. In accordance, monoclonal antibodies against

human  $\beta_2m$  usually show only a very restricted pattern of cross-reactivity against  $\beta_2m$ -homologues from other species (Brodsky et al., 1982; Teillaud et al., 1982). However, antibodies against more conserved determinants can be selected for by use of the cross-reactive capability of a  $\beta_2m$ -antiserum, e.g., the interaction between human  $\beta_2m$  and antiserum against rabbit  $\beta_2m$  (Gordon & Kindt, 1976). Such interactions can be used to construct heterologous radioimmunoassays for  $\beta_2m$ , and, in this way, Gordon and Kindt (1976) were able to detect  $\beta_2m$ -determinants in several vertebrate classes, including elasmobranchs. We used the same approach to detect  $\beta_2m$ -determinants even in invertebrates (Shalev et al., 1981). Direct binding of the antibodies to invertebrate molecules could also be demonstrated (Shalev et al., 1982).

It is hazardous to draw conclusions of evolutionary studies based solely on antibody-based cross-reactions. Ideally, the presence of  $\beta_2m$ -determinants in a given species should be confirmed by isolation and physico-chemical characterization. Such work is laborious and time-consuming, and in the meantime it would be valuable to confirm the immunological data with a functional assay. The best candidate for a functional property of  $\beta_2m$  is its binding to MHC class 1 antigen and other molecules. It can be expected that evolutionary constraint has limited diversification of the interacting sites on  $\beta_2m$  and the  $\beta_2m$ -binding molecules, respectively, much more than diversification of the other parts of the molecules. The finding that man-mouse hybrid cells express hybrid MHC/ $\beta_2m$ -molecules on the cell surface (Fellous et al., 1977; Jones et al., 1976), supports this notion. More recently, Hyafil & Strominger (1979) showed that the  $\beta_2m$ -chain of HLA-A2 antigens could be spontaneously exchanged in an equilibrium reaction with exogenous  $\beta_2m$ . Thus, we have tested the hypothesis that  $\beta_2m$  and  $\beta_2m$ -binding molecules may participate in heterologous interactions in solution.

To purify papain-solubilized rat MHC (AgB) class 1 antigens, we used spleens from inbred rat strains (Björck et al., unpublished). During purification, AgB antigens were monitored by rat  $\beta_2m$  radioimmunoassay. The purified AgB antigens were found to bind exogenously-added radiolabelled  $\beta_2m$ , as detected by immunoprecipitation of the AgB- $^{125}I$ - $\beta_2m$ -complexes with alloantiserum (II, III). Not only did the rat  $\beta_2m$  react, but also various heterologous  $\beta_2m$ s. Excess amounts of unlabelled  $\beta_2m$  from all mammalian species tested abrogated such binding, and also led to complete dissociation if added to preformed AgB- $^{125}I$ - $\beta_2m$ -complexes. Thus, the data suggest that the radiolabelled heterologous  $\beta_2m$ s bind to the AgB antigens through exchange with the originally bound homologous counterpart (rat  $\beta_2m$ ). When serially diluted, the same concentrations of different  $\beta_2m$ -homologues inhibited to the same extent the binding of  $^{125}I$ -human  $\beta_2m$  to AgB antigens. This indicates a similar binding affinity between AgB and the respective  $\beta_2m$ -homologues. Estimated with Scatchard analyses, this binding constant was in the order of  $10^9 M^{-1}$ . The heterologous interaction between  $\beta_2m$  and AgB antigens was relatively slow, about 6 (AgB 2) to 30 (AgB 3) hours for maximum binding, and markedly temperature-dependent.

Subsequently, other groups have reported similar results with heterologous  $\beta_2m$ -exchange in MHC class 1 antigens of mouse (Schmidt et al., 1981) and man (Church et al., 1982). It can be concluded that the interacting parts of  $\beta_2m$  and the MHC class 1 antigen heavy chain have been highly conserved during mammalian evolution.

The main part of  $\beta_2m$ -containing complexes in mammalian sera seems to be devoid of alloantigenic determinants (Kvist & Peterson, 1978; Natori et al., 1976; Ramanathan et al., 1982), and the possibility that they represent another kind of class 1 antigens has been discussed above. Such molecules, in sera from various mammals, were able to bind both homologous (I) and heterologous (IV)  $\beta_2m$ , probably through exchange. Again, the association process was relatively slow, markedly temperature-dependent, and apparently had equal binding affinity for homologous and heterologous  $\beta_2m$ . The capability of serum to bind heterologous  $\beta_2m$  was also demonstrated by another group (Vincent & Revillard, 1981; Vincent et al., 1981).

Lymphocytes can also bind both homologous (Hyafil & Strominger, 1979) and heterologous (Anderson et al., 1975)  $\beta_2m$ . At least part of this binding seems to be due to  $\beta_2m$ -exchange in the cell surface MHC class 1 antigens (Hyafil & Strominger, 1979; Hester et al., 1979), but other molecules have also been implicated (Sege & Peterson, 1980; Sege et al., 1979).

The binding of heterologous  $\beta_2m$  both to soluble MHC antigens and to cells can be used to detect conservative  $\beta_2m$ -determinants (II, Lögdberg et al., 1981). Invertebrate tissue extracts, that inhibited heterologous  $\beta_2m$ -radioimmunoassays and that directly bound anti- $\beta_2m$  antibodies, also inhibited the heterologous interaction between  $\beta_2m$  and AgB antigens, and between  $\beta_2m$  and lymphocytes (Shalev et al., 1981, 1982).

The evolutionary conservation of the interaction between  $\beta_2m$  and  $\beta_2m$ -binding molecules should be useful, not only in the detection of  $\beta_2m$ -determinants, but also for studies on the evolution of  $\beta_2m$ -binding molecules. Thus, we investigated the  $^{125}I$ -human  $\beta_2m$ -binding capacity of sera from various vertebrates.  $\beta_2m$ -binding glycoproteins were detected in all sera, and those of codfish were subjected to further studies (V). Many properties of the heterologous interaction between mammalian  $\beta_2m$  and codfish serum resembled those of the corresponding interactions in mammals. Also, codfish splenocytes possessed  $\beta_2m$ -binding capacity. This raises the possibility that the  $\beta_2m$ -binding glycoproteins in codfish serum are products shedded from the cell surface that could be homologous to class 1 antigens of mammals. Again, the heterologous interaction could be used for detection of " $\beta_2m$ -determinants" in invertebrate extracts (cf. Shalev et al., 1983). In this context it should also be mentioned that bacteria (Kronvall et al., 1978) and protozoa (Torpier et al., 1979) apparently have cell surface " $\beta_2m$ -receptors".

To conclude, it seems that the binding sites for each other on  $\beta_2m$  and  $\beta_2m$ -binding molecules, respectively, are protected from diversification by evolutionary forces. Thus, the heterologous interaction between these molecules promises to be a valuable tool in the continued study of their evolution. This kind of interaction could also be of a more general use in the biochemical and functional studies of class 1 antigens. For instance, it offers a specific method of radiolabelling class 1 antigens in a mixture of macromolecules. The next chapter shows another application.

### Additional class 1 alloantigens in the rat (III).

Until recently, there was evidence for only one class 1 sublocus (AgB-A) in the rat MHC (Fig. 1). However, amino acid sequence data (Blankenhorn et al., 1978), sequential immunoprecipitation with alloantisera (Natori et



al., 1979; Sporer et al., 1979) or with monoclonal antibodies (Misra et al., 1982), and genetical data (Kunz et al., 1982), indicate multiple class 1 alloantigens. In addition new data demonstrate that the AgB-C sublocus encode class 1 alloantigens with a restricted tissue distribution (Haustein et al., 1982; Stock & Günther, 1982). These antigens were therefore suggested to be homologous to the mouse Qa antigens.

Thus, we investigated whether antisera directed against products from different AgB subloci would react with our AgB-<sup>125</sup>I-human  $\beta_2$ m-complexes. A strong antiserum against AgB-C antigens failed to precipitate significant amounts of these complexes, whereas an antiserum, probably reacting with both AgB-A and AgB-C antigens clearly bound the complexes. Moreover, at saturating concentrations, a monoclonal antibody against AgB-A products could only precipitate somewhat less than 50 % of the AgB- $\beta_2$ m-complexes. This indicates that, apart from the AgB-A and AgB-C, there is at least one additional class 1 sublocus, perhaps identical to the AgB-E proposed by Kunz et al. (1982). However, our data do not answer the question as to whether the additional class 1 alloantigens are MHC-linked or not.

#### Effects of antibodies against $\beta_2$ -microglobulin on lymphocyte transformation in animal models (VI).

Antibodies against  $\beta_2$ m have multiple effects on human lymphocyte transformation (see Table 2). Only limited information exists concerning the level(s) of these effects. The inhibition of the mixed lymphocyte reaction (MLR) is exerted at the level of the responding T-lymphocytes (Lindblom et al., 1974; McCalmon et al., 1975 a,b; Östberg et al., 1976; Vincent et al., 1975). Similarly, in antigen-induced lymphocyte transformation, anti- $\beta_2$ m probably interferes with the responding T-lymphocytes, as preincubation of antigen-pulsed macrophages with anti- $\beta_2$ m does not alter their subsequent ability to present antigen to T-lymphocytes (McCalmon et al., 1980).

To delineate these effects further, it is an advantage to use animals, preferably species where inbred strains and extensive knowledge about lymphocyte subpopulations are available, such as mouse, rat, or guinea pig. The first step in this direction is to see if the human results can be extended to other species. When this study began, we had antiserum against only one animal homologue of  $\beta_2$ m, namely rabbit  $\beta_2$ m (Björck et al., 1977 a), but we later produced the corresponding guinea pig and rat reagents (Cigén et al., 1978; paper I).

Antibodies against guinea pig  $\beta_2$ m were able to interfere both with antigen- or mitogen-induced lymphocyte transformation (VI). In contrast to the human experiments where only PPD was used as antigen, we extended the inhibitory effect on antigen-stimulation to other soluble antigens. Lymphocyte transformation with mitogens thought to selectively stimulate T-lymphocytes (ConA, PHA), was clearly inhibited by the anti- $\beta_2$ m. On the other hand, anti- $\beta_2$ m did not inhibit stimulation induced by two substances reported in other species to be selective B-lymphocyte mitogens (DxS, LPS). Anti- $\beta_2$ m did not consistently enhance background <sup>3</sup>H-thymidine-uptake. However, in a separate study, using the same antiserum but a different culture system and usually partly purified lymphocyte populations, the anti- $\beta_2$ m exerted strong stimulation on spontaneous lymphocyte transformation of a T-lymphocyte subpopulation (Yamashita et al., 1979). Thus, all the guinea pig data are compatible with  $\beta_2$ m-involvement in a common

step of T-lymphocyte activation, e.g., in the interleukin interactions.

In unpublished experiments, we have seen profound inhibitory effects of anti- $\beta_2m$  on various kinds of lymphocyte transformation in rabbit or rat cultures, respectively. Goat anti-rabbit  $\beta_2m$  also gave pronounced increment of spontaneous  $^3H$ -thymidine-uptake in rabbit lymphocytes (Fig. 5).

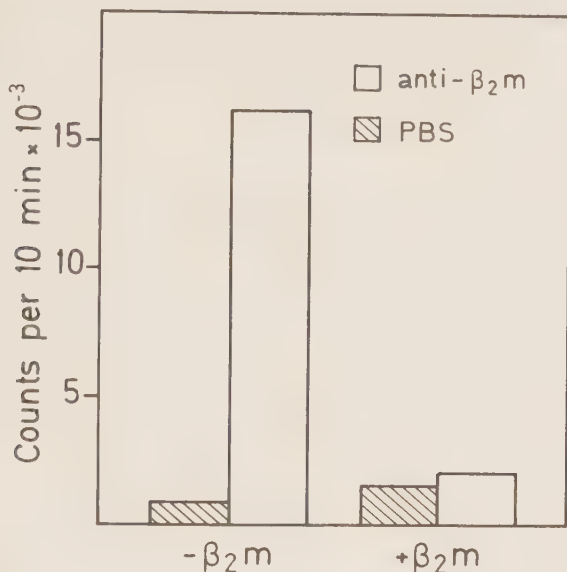


Figure 5

Stimulation of thymidine-uptake in rabbit lymphocytes by anti- $\beta_2m$ -microglobulin antibodies. Rabbit peripheral blood lymphocytes were cultured essentially as described for guinea pig lymphocytes in paper VI. Goat anti-rabbit  $\beta_2m$  IgG were added to a final concentration of 2 ml/ml. Control cultures received an equal volume of phosphate buffered saline (PBS). Some of the cultures also contained 40  $\mu g/ml$  of purified rabbit  $\beta_2m$ . Thymidine-uptake was measured on day 3.

To summarize, the effects of anti- $\beta_2m$  on lymphocyte transformation are not a peculiarity to human peripheral blood lymphocytes, but are probably valid in most mammals. However, the recorded effects may vary between lymphocyte subpopulations, different culture systems, and different antisera. The last item is emphasized by recent studies with various monoclonal antibodies against  $\beta_2m$  (Stern et al., 1982, Fed. Proc. 41, 598, abstract), which showed that such antibodies could differ distinctly in their pattern of effects in lymphocyte transformation and rosette tests.

#### Effect of antibodies against $\beta_2m$ -microglobulin on natural killer cell-mediated cytotoxicity (VII).

A minor subpopulation of lymphocyte-like cells - the natural killer (NK) cells - is thought to play a major role in tumor surveillance (cf. Herberman, 1982). The NK cells, which are cytotoxic without apparent prior sensitization, may represent a phylogenetically old defence system (cf.

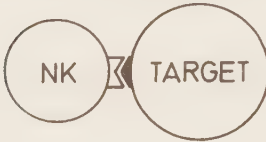


Cooper, 1980). It was therefore of interest to investigate the potential involvement of  $\beta_2m$  in NK-activity.

Antibodies against human  $\beta_2m$  were found to inhibit NK-activity. This effect was related to the target cells, i.e., anti- $\beta_2m$ -pretreatment of target cells inhibited subsequent NK-activity, whereas pretreatment of the NK cells did not have any major effect. Moreover, the antibodies did not interfere with target binding, but markedly reduced target lysis (Fig. 6). In this context, the lytic phase is defined as all events taking place after formation of NK-target cell conjugates (target binding), until the final destruction of the target cell.

1. TARGET BINDING:

Not affected by  
anti -  $\beta_2m$

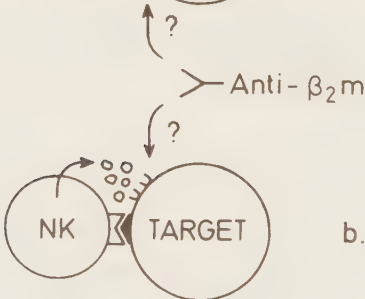


2. TARGET LYSIS:

Inhibited at the  
level of the target  
cells by anti- $\beta_2m$



a. NK cell activation



b. Target cell destruction

Figure 6

Antibodies against  $\beta_2$ -microglobulin inhibit the cytotoxic activity of natural killer cells. See text for explanation.

Target cell structures are probably involved in two separate steps during the lytic phase (Fig. 6:2). First, after conjugate formation, the NK cells are very rapidly activated. This activation is presumably due to triggering of a receptor on the NK cells (Fig. 6:2a). In a minimal hypothesis, this receptor is the same as the target binding structure. If this is true, blocking of target trigger molecules would also inhibit target binding. Thus, a more plausible level for the inhibitory effect of anti- $\beta_2m$  is at the second step that involve target cell structures, namely the delivery of the lytic message from the NK cell to a presumed acceptor site on the target cell (Fig. 6:2b). Recent studies implicate soluble cytotoxins - lymphotoxin (LT) - and proteases as important factors in the "lethal hit" (cf. Herberman, 1982). Thus, the lytic signal may be mediated by a complement-like system, which could bind to an immunoglobulin-like ( $\beta_2m$ ) acceptor on the target cells.

Goding & Walker (1980) postulated a similar lytic system for CTL-mediated lysis. Indeed, other kinds of cell-mediated cytotoxicity have also been reported to involve LT (Kondo et al., 1981; Sawada et al., 1980; cf. Ware & Granger, 1981), and to be inhibited by anti- $\beta_2m$  (see Table 2). It is notable that the "medial" histocompatibility antigens, targets for unrestricted CTL, are largely  $\beta_2m$ -associated (Lindahl & Langhorne, 1981).

In conclusion, we suggest that  $\beta_2m$  or closely associated structures are the acceptor site for the lytic signal from NK cells. This may also be relevant for other types of cell-mediated cytotoxicity.

## SUMMARY

Rat  $\beta_2m$  was isolated from the urine of animals with sodium chromate induced tubular damage. The protein was purified by sequential use of gel chromatography, ion exchange chromatography, zone electrophoresis, and finally repeated gel chromatography. The physico-chemical properties of rat  $\beta_2m$  were similar to those of other  $\beta_2m$  homologues. Urinary rat  $\beta_2m$  also existed as a minor form with lower pI. Rat  $\beta_2m$  displayed extensive immunological cross-reactivity with purified  $\beta_2ms$  from other mammals. In rat serum,  $\beta_2m$  was found in high molecular weight complexes and in free form.

Purified rat MHC class 1 antigens (AgB) were able to bind exogenous rat  $\beta_2m$ , most probably through an exchange reaction. Similarly, the AgB antigens bound  $\beta_2m$  homologue of other mammalian species. Some characteristics of these heterologous interactions were determined. The affinity was of the same order regardless whether homologous (rat) or heterologous  $\beta_2m$  was bound. Scatchard analysis of the interaction between AgB 2 antigens and  $^{125}I$ -human  $\beta_2m$  gave a value for the association constant of  $3 \times 10^9 M^{-1}$ . Moreover, the  $\beta_2m$  subunit of high molecular weight complexes in mammalian sera apparently could be exchanged for exogenously-added heterologous  $\beta_2m$ . These data indicate a high degree of conservation of the interacting sites on  $\beta_2m$  and  $\beta_2m$ -binding molecules, respectively. Thus, such interactions can be used in evolutionary studies to detect conserved  $\beta_2m$ -determinants. In this context, it was not surprising to find glycoproteins in fish serum capable of reacting with a highly conserved part of different mammalian  $\beta_2ms$ . These fish molecules could be homologous to mammalian class 1 antigens.

Heterogeneity of AgB 2 antigen- $^{125}I$ -human  $\beta_2m$  complexes was indicated by immunoprecipitation with different anti-AgB antisera, suggesting that inbred rats produce more than one type of class 1 alloantigen.

Antibodies against guinea pig  $\beta_2m$  reduced the proliferative response of guinea pig lymphocytes to stimulation with mitogens or soluble antigens, which generalizes similar results previously found in human systems.

Anti- $\beta_2m$  antibodies strongly inhibited human natural killer-activity. The antibodies appear to operate on the target cells, and interfere selectively with the lytic phase. It is suggested that  $\beta_2m$  may be involved as a target cell acceptor for the lytic message from natural killer cells.

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## RAT $\beta_2$ -MICROGLOBULIN. ISOLATION, PROPERTIES AND RELATION TO $\beta_2$ -MICROGLOBULINS FROM OTHER SPECIES

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**Abstract**—Rat  $\beta_2$ -microglobulin was isolated from the urine of animals with sodium-chromate induced tubular damage. It was purified by sequential use of gel chromatography, ion exchange chromatography, zone electrophoresis, and finally repeated gel chromatography. The rat protein was similar to human  $\beta_2$ -microglobulin in properties such as molecular weight and the presence of an apparently analogous disulfide loop. Electrophoretically, however, it migrated in the  $\gamma$ -globulin region, which was reflected in a higher pI-value (pH 7.4). A minor rat  $\beta_2$ -microglobulin form with a somewhat lower pI (pH 6.8) was found. On both Ouchterlony immunodiffusion analyses and radioimmunoassays, rat  $\beta_2$ -microglobulin was found to cross-react with purified  $\beta_2$ -microglobulins from four other species: rabbit, guinea pig, mouse, and man. The gel chromatography profile of  $\beta_2$ -microglobulin in rat serum was investigated by radioimmunoassay.  $\beta_2$ -microglobulin was found in high mol. wt complexes and in free form.

### INTRODUCTION

Human  $\beta_2$ -microglobulin ( $\beta_2$ m) is a low mol. wt protein consisting of a single polypeptide chain with 100 amino acid residues (Berggård & Bearn, 1968). It has been shown to be produced by most cell types studied (Nilsson *et al.*, 1973) except mature erythrocytes (Evrin & Pertoft, 1973) and lymphoma cells of the Daudi cell line (Nilsson *et al.*, 1974). It can be detected bound to cell surfaces (Peterson *et al.*, 1972; Poulik, 1973) and in various body fluids (Berggård & Bearn, 1968).

Human  $\beta_2$ m has aroused interest mainly because of its immunological significance. It is structurally related to the constant domains of the immunoglobulins (Peterson *et al.*, 1972; Smithies & Poulik, 1972). Furthermore, the small sub-unit of the major serologically-defined histocompatibility antigens (HLA) in man has been shown to be  $\beta_2$ m (Grey *et al.*, 1973; Nakamuro *et al.*, 1973; Peterson *et al.*, 1974). Antibodies directed against human  $\beta_2$ m have been reported to interfere with the cellular response in immunological *in-vitro* reactions (Bach *et al.*, 1973; Möller & Persson, 1974). Because of these studies, a connection between  $\beta_2$ m and lymphocyte receptor structures was postulated. Recently, a lymphokine activity—human colony-stimulating activity—was shown to disappear after treatment with anti- $\beta_2$ m (Price *et al.*, 1976).

It is also of interest that the serum level of  $\beta_2$ m is increased in certain diseases. Elevated serum  $\beta_2$ m is a sensitive indicator of renal failure (Bernier *et al.*, 1968; Peterson *et al.*, 1969). Serum  $\beta_2$ m also increases in many patients with neoplastic and autoimmune conditions (Evrin & Wibell, 1973).

To obtain more information about the biological role and evolution of  $\beta_2$ m, it became necessary to work in animal systems. So far, homologues of  $\beta_2$ m have

been isolated from dog (Smithies & Poulik, 1972), rabbit (Berggård, 1974), mouse (Natori *et al.*, 1975), guinea pig (Cigén *et al.*, 1978), cow (Groves & Greenberg, 1977), and chicken (Winkler & Sanders, 1977). The major histocompatibility antigens of several of these species were shown to be coupled to the  $\beta_2$ m (cf. Björck *et al.*, 1977). In the mouse,  $\beta_2$ m is reported to be associated not only with the major histocompatibility antigens (H-2) but also with the structurally and genetically related TL- (Östberg *et al.*, 1975; Vitella *et al.*, 1975) and Qa-2- (Michaelson *et al.*, 1977) antigens; with the H-Y-antigen (Fellous *et al.*, 1978), and with a cell-cell interaction factor (Armerding *et al.*, 1975).

This paper reports the purification and some characteristics of rat  $\beta_2$ m. Its relations to other  $\beta_2$ ms were investigated by immunochemical methods and by comparisons of amino acid compositions. We also analysed  $\beta_2$ m and  $\beta_2$ m-containing molecules in rat serum.

Isolation of rat  $\beta_2$ m was previously reported by Poulik, who published brief notes on his purification schedule (Callahan *et al.*, 1976) and on the NH<sub>2</sub>-terminal sequence of the protein (Uhr *et al.*, 1976).

### MATERIAL AND METHODS

#### Rats

Outbred albino (Sprague-Dawley) rats from local dealers, weighing about 200 g were used.

#### Urine and sera

Female rats (10–40 each time) were injected sub-cutaneously with sodium chromate (single dose of 20 mg/kg) to induce tubular damage in the kidneys. Urine was collected in 24-hr portions in vessels containing 0.5 ml of 5% sodium azide as preservative. Each 24-hr collection was pooled, the sodium azide concentration was adjusted to 0.1% and pH was adjusted to between 6 and 7. The urine portions were paper-filtered and stored at  $-20^{\circ}\text{C}$ . Urine was collected for five

\* Deceased June 1978.

days and the protein content estimated with Uristix strips (Ames Company, Slough, England). The Uristix test was usually negative in normal urine but positive after sodium chromate administration. Maximum proteinuria ( $2+3+$ ) was usually found on day 2–4. In most cases, several rats died during the collection period.

In three small groups of rats (7, 6 and 6, respectively) the urinary  $\beta_2$ m excretion was monitored by radioimmunoassay as soon as specific antibodies against rat  $\beta_2$ m were obtained. The daily excretion,  $\mu\text{g}$  rat  $\beta_2$ m/day per rat, was  $1.6 \pm 0.7$ ,  $1.8 \pm 0.4$  and  $2.1 \pm 0.3$  on three consecutive days. After sodium chromate injections, the daily excretion increased and reached a maximum of  $640 \pm 70$  on day 3, an increase with a factor of about 400. The values are means  $\pm$  S.E.M. Sera were obtained from healthy rats by cardiac puncture. The sera were stored at  $-20^\circ\text{C}$  until used.

#### Purified $\beta_2$ ms

Human  $\beta_2$ m (Berggård & Bearn, 1968), rabbit  $\beta_2$ m (Berggård, 1974), guinea pig  $\beta_2$ m (Cigén *et al.*, 1978) and mouse  $\beta_2$ m (B. Åkerström & I. Berggård, unpublished results) were purified in this laboratory.

#### Antisera

Three rabbits and a goat were immunized with purified rat  $\beta_2$ m by earlier-described immunizing schedules (Berggård & Bearn, 1968; Björck *et al.*, 1977). The monospecificity of the antisera obtained was tested on immunoelectrophoresis against concentrated rat urinary proteins. Three goat anti-rabbit  $\beta_2$ m sera (Berggård, 1974), a rabbit anti-human  $\beta_2$ m serum (Berggård & Bearn, 1968), a goat anti-human  $\beta_2$ m serum (raised as in Björck *et al.*, 1977), a rabbit anti-mouse  $\beta_2$ m serum (B. Åkerström & I. Berggård, unpublished results), and a goat anti-guinea pig  $\beta_2$ m serum (Cigén *et al.*, 1978) were also used.

#### Techniques used in purification

**Ultrafiltration.** Urinary and partly-purified rat  $\beta_2$ m was concentrated by ultrafiltration with 23/32-inch dialysis tubing (Union Carbide Corp., Chicago, IL), which completely retains  $\beta_2$ m under the conditions used (Berggård & Bearn, 1968).

**Gel filtration.** Carried out in Sephadex G-100 or G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) with 0.02 M Tris-HCl buffer + 0.15 M sodium chloride + 0.02% sodium azide, pH 8.1, as eluant.

**Ion exchange chromatography** on DEAE-cellulose 23-SH (Serva Feinbiochemia, Heidelberg, Germany) was performed with linear salt gradient elution. The mixing vessel contained 750 ml of 0.02 M Tris-HCl buffer, pH 8.1, and the reservoir vessel 740 ml of the same buffer with 0.1 M sodium chloride.

**Zone electrophoresis.** Carried out on blocks of Pevecon C-870 (Kema-Nord AB, Stockholm, Sweden) (Berggård & Bearn, 1968) with 0.1 M borate buffer, pH 8.9. The electrophoresis was continued for about four days with an electric field of 4 V/cm.

**Isoelectric focusing.** Conducted at  $5^\circ\text{C}$  in a 110-ml column (LKB-Produkter AB, Bromma, Sweden), (Vesterberg, 1971). A pH-gradient, range 5–8, was created using carrier ampholyte solutions (Ampholine, LKB-Produkter AB, Bromma, Sweden) and stabilized by a sucrose gradient from 0 to 50%.

#### Immunochemical techniques

Double immunodiffusion, single radial immunodiffusion and micro-immunoelectrophoresis were carried out as previously described (Berggård & Bearn, 1968). Purified preparations of rat  $\beta_2$ m were used as standard in the single radial immunodiffusions. Radioimmunoassay of  $\beta_2$ ms was performed using the polyethylenglycol-exclusion method (PEG 6000, Kebo AB, Stockholm, Sweden) described by Plesner (1975).  $\beta_2$ ms were  $^{125}\text{I}$  (Amersham, England)-radiolabelled by the Chloramin T method (Greenwood *et al.*,

1963).  $^{125}\text{I}$  was counted in a  $\gamma$ -counter, inhibition curves were made by plotting counts/min against the log of the concentration.

#### Other electrophoretic techniques

Agarose gel electrophoresis was carried out in 0.075 M sodium barbital buffer, pH 8.6, containing 0.002 M calcium lactate (Johansson, 1972). Polyacrylamide gel electrophoresis in a discontinuous buffer system and starch gel electrophoresis in 8 M urea and formate buffer, pH 3.0, were run as described elsewhere (Peterson & Berggård, 1971). The protein samples to be analysed by starch gel electrophoresis were dissolved in 0.2 M Tris-HCl buffer, pH 8.0, or in the same buffer with 8 M urea. Some of these samples were reduced with 0.1 M 2-mercaptoethanol for 60 min at room temperature and thereafter alkylated with 0.12 M iodoacetamide for 30 min. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (King & Laemmli, 1971), was used for determination of mol. wt with human  $\beta_2$ m and light and heavy chains of IgG as reference proteins. For analytical isoelectric focusing on thin layer polyacrylamide gel, we used Ampholine PAG plate (LKB-Produkter AB, Bromma, Sweden) with a pH-gradient from 3.5 to 9.5. Crossed immunoelectrophoresis was performed in agarose gels essentially as described by Laurell (1965).

#### Protein determinations

Protein concentrations were measured by the modified Folin method of Lowry *et al.* (1951) with human IgG as standard, or in more purified fractions by reading the absorbance at 280 nm with purified rat  $\beta_2$ m as standard. The absorption coefficient of rat  $\beta_2$ m at 280 nm and its light-absorption spectrum were determined in 0.1 M sodium phosphate buffer on a Gilford Model 222 spectrophotometer, after correction for ash and moisture. Moisture was measured by drying to constant weight at  $105^\circ\text{C}$  and 0.05 mm Hg.

#### Determination of mol. wt by gel chromatography

Analyses were made on a Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) column ( $120 \times 1.5$  cm), equilibrated with 0.05 M sodium acetate buffer, pH 4.8, containing 6 M guanidine hydrochloride (Mann & Fisch, 1972), with heavy and light chains of human IgG, bovine albumin, ovalbumin, equine myoglobin and human  $\beta_2$ m as reference proteins. Rat  $\beta_2$ m and the reference proteins were reduced for 2 hr in 0.2 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride and 0.02 dithioerythritol. After this, iodoacetamide was added to a concentration of 0.05 M, and the samples were left to alkylate for 30 min in the dark.

#### Ultracentrifugation

Sedimentation-equilibrium ultracentrifugation was carried out in a MSE-75 Centriscan using the meniscus depletion technique of Yphantis (1964). The protein to be investigated was used in a dilution of 0.2 mg/ml in 0.02 M Tris-HCl buffer with 0.15 M NaCl and 6 M guanidine hydrochloride. Guanidine hydrochloride was present in the solvent in order to avoid complications caused by possible self-association of the protein. After 5 hr of centrifugation at 55,000 rev/min followed by 22 hr at 40,000 rev/min the system was in equilibrium and recordings were made. The effects of non-ideality as observed were negligible. A partial specific volume for the calculation of molecular weight was estimated from the amino acid composition as described by Cohn and Edsall (1943).

#### Amino-acid analysis

Quantitative amino-acid analyses were done with the use of a Durrum Model D-500 amino-acid analyser according to the instructions supplied by Durrum, Palo Alto, CA. The protein samples (about 0.5 mg) were hydrolyzed in 6 M HCl at  $110^\circ\text{C}$  for 24 and 72 hr (von Hojsten *et al.*, 1965). Half-

cystein was measured as cysteic acid after performic acid oxidation (Hirs, 1967). Tryptophan was estimated spectrophotometrically (Edelhoch, 1967).

#### Serum separation

Serum was separated on a Sephadex G-200 column ( $90 \times 1$  cm) equilibrated with 0.02 M Tris-HCl buffer + 0.15 M sodium chloride + 0.02% sodium azide, pH 8.1, on a DEAE-cellulose column ( $2 \times 10$  cm) equilibrated with the same buffer lacking sodium chloride (eluted with a linear salt-gradient from 0 to 0.5 M sodium chloride), or on agarose gel electrophoresis. The eluant from the columns was analysed for  $\beta_2$ m content by radioimmunoassay. The agarose gel was sliced and each slice frozen in test tubes with the same amount of 0.15 M sodium chloride. After 24 hr the samples were thawed, and the  $\beta_2$ m concentration in the sodium chloride was measured by radioimmunoassay.

## RESULTS

### Purification

$\beta_2$ m was purified at 4°C from the urine of rats with induced tubular damage according to the following procedure. As a first step, concentrates of urinary proteins were chromatographed on columns of Sephadex G-100. Figure 1 shows a representative graph from such a separation. In the first preparation, when antisera against rat  $\beta_2$ m were not available, we pooled material with the elution volume of human  $\beta_2$ . Specific antisera were used to trace rat  $\beta_2$ m in later experiments (Fig. 1). With few exceptions, the curve of total protein did not show a distinct peak at the position of  $\beta_2$ m. With radioimmunoassay, we saw an additional small peak reacting with anti-rat  $\beta_2$ m, not detected by single radial immunodiffusion, apparently similar in size to the major high mol. wt material in rat serum that reacts with anti-rat  $\beta_2$ m (see below).

The pooled material from one or several Sephadex G-100 chromatographies was concentrated and separated further by ion exchange chromatography on DEAE-cellulose. A linear salt-gradient was used for elution. Figure 2 shows the results of a typical experiment. The first peak after void volume contained most of the rat  $\beta_2$ m, but  $\beta_2$ m was traceable in slowly decreasing quantities also in later peaks.

The material from two or more ion exchange chromatographies was pooled, concentrated, and subjected to zone electrophoresis at pH 8.9. One distinct protein peak, containing most of the  $\beta_2$ m, could be seen. Only small amounts of contaminants seemed to be removed.

The pooled and concentrated protein from zone electrophoresis was finally subjected to gel chromatography on Sephadex G-75. A symmetrical peak with the elution volume of human  $\beta_2$ m was obtained. The main part of this peak was pooled, dialyzed exhaustively against distilled and deionized water, and lyophilized. Table 1 records one complete preparation. Different steps of purification were compared on agarose gel electrophoresis (Fig. 3).

#### Homogeneity of the isolated protein and an alternative scheme of purification

When the purified rat  $\beta_2$ m was subjected to polyacrylamide gel electrophoresis, a small amount of a more anodally migrating material could be seen. The same was true when rat  $\beta_2$ m was investigated by means

Table 1. Purification of rat  $\beta_2$ m

| Fraction                      | Total protein (mg) | $\beta_2$ m <sup>a</sup> (mg) | Yield (%) | Purification (fold) |
|-------------------------------|--------------------|-------------------------------|-----------|---------------------|
| Concentrated urinary proteins | 3800 <sup>b</sup>  | 87                            | 100       | 1                   |
| Sephadex G-100 chromatography | 250 <sup>b</sup>   | 42                            | 48        | 7                   |
| DEAE-cellulose chromatography | 19 <sup>c</sup>    | 18                            | 21        | 41                  |
| Zone electrophoresis          | 10.6 <sup>c</sup>  | 10.3                          | 12        | 42.5                |
| Sephadex G-75 chromatography  | 6.4                | 6.4                           | 7.5       | 44                  |

<sup>a</sup> Measured by the single radial immunodiffusion technique.

<sup>b</sup> Determined by the Folin technique.

<sup>c</sup> Estimated from the O.D. at 280 nm.

of agarose gel electrophoresis (Fig. 3). For this reason, crossed immunoelectrophoresis of rat  $\beta_2$ m was performed. Both the strong and the weak band reacted with our goat anti-rat  $\beta_2$ m in what appeared to be a reaction of at least partial identity. The concentration difference between the two materials, however, was large, and the results were somewhat difficult to interpret.

The purified rat  $\beta_2$ m was also analysed by isoelectric focusing on polyacrylamide gels. It was found to take colour (Coomassie) less effectively than other  $\beta_2$ ms, but two distinct bands were seen at pI values of 6.6 and 7.2, respectively. The major band had a pI of 7.2. Human  $\beta_2$ m investigated on the same plates separated into two main bands at pI values of 5.4 and 5.8, respectively. Because of these observations, we introduced isoelectric focusing on a sucrose-gradient column as a third purification step, replacing the block electrophoresis, for a small preparation. Rat  $\beta_2$ m was traced by radioimmunoassay (RIA), instead of the single radial immunodiffusion used above, to increase the sensitivity. As Fig. 4 shows, almost all the material was focused in a sharp peak at pI of 7.4. With RIA, an additional small peak (about 1.5% of the total amount of  $\beta_2$ m) was found at a pI value of 6.8. This peak could completely inhibit the binding of  $^{125}$ I-rat  $\beta_2$ m by goat anti-rat  $\beta_2$ m, indicating that the inhibition was specific. Furthermore, dilution series of the major and the minor peak gave parallel inhibition curves. Therefore, urinary rat  $\beta_2$ m apparently consists of at least two forms.

#### Other properties of the major component

**Amino-acid composition.** The amino-acid composition of rat  $\beta_2$ m is presented in Table 2. The analyses were made on purified material presumably containing the minor component, but in low quantities that would not noticeably influence the values. Calculations were based on the assumption of 100 amino-acid residues per molecule of rat  $\beta_2$ m.

The amino acid composition of rat  $\beta_2$ m was compared with that of other  $\beta_2$ ms by the index  $s\Delta n = \frac{1}{2} \sum (n_{iA} - n_{iB})^2$  which is an unbiased estimator of the number of loci at which two sequences of the same length are different (Cornish-Bowden, 1977).  $n_{iA}$  and  $n_{iB}$  are the number of residues of the  $i$ th type of amino



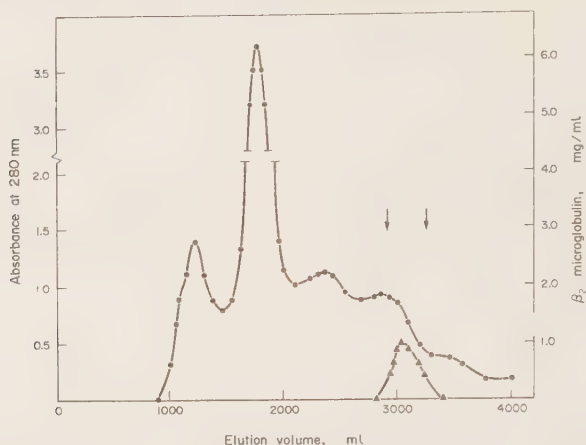


Fig. 1. Chromatography on Sephadex G100 of concentrated urinary proteins from rats with sodium chromate-induced tubular damage. The column ( $5 \times 105$  cm) was equilibrated with  $0.02$  M Tris-HCl buffer, pH 8.1, containing  $0.15$  M NaCl and  $0.02\%$  sodium azide. The flow rate was  $43$  ml/hr. The applied sample ( $17$  ml) contained  $1750$  mg of total protein and  $63$  mg of  $\beta_2$ m. Total protein was measured at O.D.<sub>280</sub> (●—●).  $\beta_2$ m was determined by the single radial immunodiffusion technique (▲—▲). Arrows indicate the pooled material.

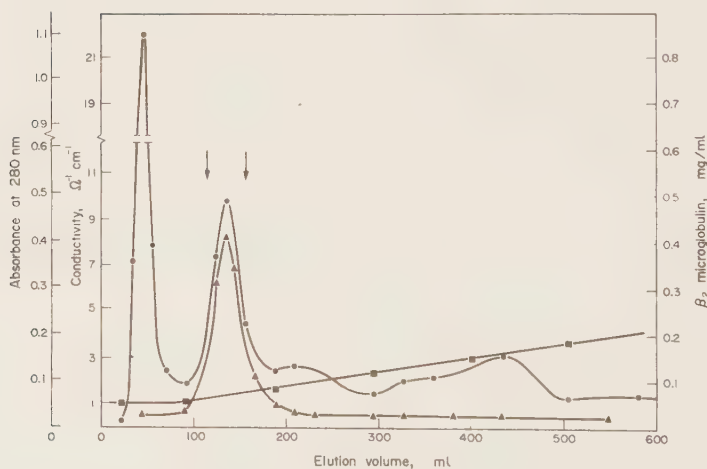


Fig. 2. Ion exchange chromatography on DEAE-cellulose of pooled  $\beta_2$ m-containing material from gel chromatography (Fig. 1). A sample ( $11.5$  ml) containing  $250$  mg of total protein and  $48$  mg of  $\beta_2$ m was dialyzed against  $0.02$  M Tris-HCl buffer, pH 8.1, and applied to a column ( $2 \times 10$  cm) of the ion exchanger equilibrated with the same buffer. The protein sample was eluted with a linear sodium chloride gradient as described in materials and methods. Total protein was measured at O.D.<sub>280</sub> (●—●). The conductivity was measured to check the gradient (■—■), and  $\beta_2$ m was traced by the single radial immunodiffusion technique (▲—▲). The pooled material is indicated by arrows.



Fig. 3. Agarose gel electrophoresis: Comparison of different purification steps (1-3). Electrophoretic characterization of rat  $\beta_2$ m (3-6): (1) concentrated rat urinary proteins; (2) concentrated  $\beta_2$ m pool after separation of urinary proteins on Sephadex G-100 (see Fig. 1); (3) purified rat  $\beta_2$ m; (4) pool of serum from 100 rats; (5) Human plasma; (6) Purified human  $\beta_2$ m.

acid in proteins A and B, respectively. Table 3 gives the result of this comparison.

**Presence of intra-chain disulfide loop.** When investigated on starch gel electrophoresis, the mobilities of both rat and human  $\beta_2$ m decreased by approximately the same extent after reduction and alkylation in the presence of urea (Fig. 5). If the proteins were subjected to the same pretreatment without the presence of urea, no decrease in mobility was seen. This suggests that the two proteins have an intrachain disulfide loop of similar size and that the loop is reduced only in the presence of a denaturing agent.

**Molecular weight.** The molecular weight of rat  $\beta_2$ m was indistinguishable from that of human  $\beta_2$ m when measured by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. It was 12,300 when measured by gel chromatography in 6 M guanidine-HCl. The value calculated from amino acid compositions was 11,750. From sedimentation-equilibrium data and an estimation of the partial specific volume—0.734 ml/g—a mol. wt of 11,700 was calculated.

**Mobility in agarose gel.** The mobility on agarose gel electrophoresis at pH 8.6 was that of a  $\gamma$ -globulin (Fig. 3).

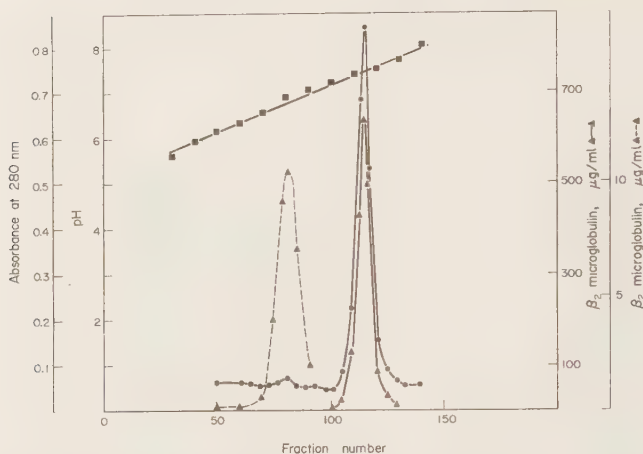


Fig. 4. Isoelectric focusing on sucrose gradient column: about 5 mg of rat  $\beta_2$  from the second purification step (ion exchange chromatography) was applied to the column. The focusing proceeded for 24 hr at a constant power of 12 W and a current of 10–1.5 mA. The column was fractionated and then analysed for pH (■—■), for total protein by measuring the absorbance at 280 nm (●—●), and for rat  $\beta_2$  by radioimmunoassay (▲—▲, ▲—▲). Note that the two rat  $\beta_2$  peaks are plotted at different scales.

**Absorption coefficient.** The absorption coefficient at 280 nm and pH 7.0 was estimated as  $1.42 \times 10^4 M^{-1}$  with a value of 11,800 for the mol. wt. When calculated from the amino-acid composition according to Wetlaufer (1962), the value was  $1.62 \times 10^4 M^{-1} \text{ cm}^{-1}$ .

#### Cross-reactions

The rabbit anti-rat  $\beta_2$  serum with the highest titre

and avidity was found to precipitate purified  $\beta_2$ ms from guinea pig and mouse on Ouchterlony double diffusion plates. It could not precipitate human  $\beta_2$ m, nor could high concentrations of human  $\beta_2$ m interfere with its precipitation of rat  $\beta_2$ m. Rat  $\beta_2$ m and guinea pig  $\beta_2$ m gave a reaction of partial identity (Fig. 6). The cross-reaction between rat and mouse  $\beta_2$ m was not further investigated because of shortage of the latter. Two different bleedings of the goat anti-rat  $\beta_2$ m



Fig. 5. Starch gel electrophoretic analysis in 8 M urea-formate buffer, pH 3.0, of (1) Rat  $\beta_2$ m, (2) Human  $\beta_2$ m. Pretreatment: (a) no treatment, (b) reduced with 2-mercaptoethanol and alkylated with iodoacetamide in the presence of urea, (c) treated with 2-mercaptoethanol and iodoacetamide, as in (b), without the presence of urea. See text for explanations.

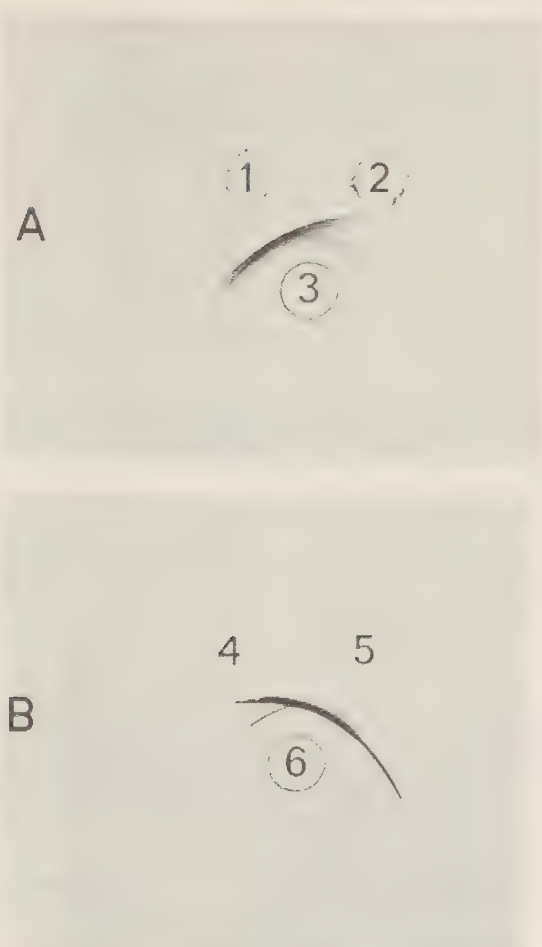


Fig. 6. Ouchterlony immunodiffusion analyses. Dried gels were stained with Coomassie Brilliant Blue. (A) Partial identity between rat and guinea pig  $\beta_2$ m: (1) rat  $\beta_2$ m, (2) guinea pig  $\beta_2$ m, (3) rabbit anti-rat  $\beta_2$ m antiserum. (B) Partial identity between rat and rabbit  $\beta_2$ m: (4) rat  $\beta_2$ m, (5) rabbit  $\beta_2$ m, (6) goat anti-rabbit  $\beta_2$ m antiserum.

serum was used. The early bleeding (after one booster dose) did not precipitate any of the purified human, guinea pig, or rabbit  $\beta_2$ m. The later bleeding (after four booster doses), however, precipitated all of these proteins, and reactions of partial identity with rat  $\beta_2$ m were obtained. The three goat anti-rabbit  $\beta_2$ m sera precipitated purified rat  $\beta_2$ m and gave reactions of partial identity between rat and rabbit  $\beta_2$ m (Fig. 6).

Rabbit anti-mouse  $\beta_2$ m, goat anti-guinea pig  $\beta_2$ m, goat anti-human  $\beta_2$ m, and the rabbit anti-human  $\beta_2$ m apparently did not precipitate purified rat  $\beta_2$ m.

We also used radioimmunoassay to study cross-

reactions between purified  $\beta_2$ ms. High concentrations of rabbit, guinea pig, or human  $\beta_2$ m ( $10^4$ – $10^6$  times the corresponding concentrations of rat  $\beta_2$ m) could inhibit the binding of  $^{125}\text{I}$ -rat  $\beta_2$ m to goat anti-rat  $\beta_2$ m. Heterologous binding was also tested. The results are shown in Table 4 in terms of the 50% binding titre. The titres given are comparable only within each group of labelled protein, as the radiolabelled proteins were used in different concentrations (due to differences in specific activity). Goat anti-rat  $\beta_2$ m serum from two different bleedings was used (see above). The early bleeding was much

Table 2. Amino-acid composition of rat  $\beta_2$ m

| Amino acid       | Mean $\pm$ S.E.M. <sup>a</sup> | To nearest integer |
|------------------|--------------------------------|--------------------|
| AspX             | 10.0 $\pm$ 0.09                | 10                 |
| Thr <sup>b</sup> | 8.0 $\pm$ 0.1                  | 8                  |
| Ser <sup>b</sup> | 6.2 $\pm$ 0.1                  | 6                  |
| GluX             | 13.0 $\pm$ 0.5                 | 13                 |
| Pro              | 8.9 $\pm$ 0.2                  | 9                  |
| Gly              | 2.4 $\pm$ 0.2                  | 2                  |
| Ala              | 2.2 $\pm$ 0.1                  | 2                  |
| Cys <sup>c</sup> | 2                              | 2                  |
| Val <sup>d</sup> | 5.8 $\pm$ 0.1                  | 6                  |
| Met              | 1.9 $\pm$ 0.1                  | 2                  |
| Ile <sup>e</sup> | 6.7 $\pm$ 0.2                  | 7                  |
| Leu              | 6.0 $\pm$ 0.1                  | 6                  |
| Tyr <sup>b</sup> | 3.9 $\pm$ 0.2                  | 4                  |
| Phe              | 4.8 $\pm$ 0.1                  | 5                  |
| His              | 4.1 $\pm$ 0.08                 | 4                  |
| Lys              | 8.9 $\pm$ 0.1                  | 9                  |
| Arg              | 3.1 $\pm$ 0.03                 | 3                  |
| Trp <sup>e</sup> | 2                              | 2                  |
| Total            | 99.9                           | 100                |

<sup>a</sup>Calculated on the basis of 100 amino-acid residues per molecule. Except when noted, all figures are means  $\pm$  S.E.M. obtained from analyses of three different preparations, and each analysis is an average of one 24-hr and one 72-hr hydrolysis.

<sup>b</sup>Values obtained by extrapolation to zero-hour hydrolysis.

<sup>c</sup>Measured as cysteic acid on two preparations.

<sup>d</sup>72-hr hydrolysis value only.

<sup>e</sup>Determined spectrophotometrically on one preparation.

weaker than the later bleeding. This is also reflected in much weaker cross-reactions.

#### $\beta_2$ m in rat serum

The content of rat  $\beta_2$ m in rat serum was measured with radioimmunoassay. The measuring range of the radioimmunoassay was 2–20  $\mu$ g/l; the assay had a standard deviation of 8% as calculated from double values in this range (50 d.f.). Inhibition curves made with dilution series of rat serum or purified rat  $\beta_2$ m were parallel. Eleven individual rat sera from apparently healthy rats were analysed for rat  $\beta_2$ m and gave a value of  $5.3 \pm 0.5$   $\mu$ g/ml (mean  $\pm$  S.D.).

Rat serum was separated on a Sephadex G-200

column, and the eluent was analysed for rat  $\beta_2$ m by radioimmunoassay. At least three different  $\beta_2$ m-containing peaks were seen (Fig. 7). Most of the rat  $\beta_2$ m (about 75%) was eluted at the position of free  $\beta_2$ m. The other major peak eluted slightly after the last protein peak that gave measurable absorbance at 280 nm. Inhibition curves with material from the 'free  $\beta_2$ m' peak were parallel to inhibition curves with purified rat  $\beta_2$ m, whereas material from the major high mol. wt  $\beta_2$ m peak gave inhibition curves with a somewhat flatter slope. This indicates that the measurement of the latter peak is only semi-quantitative. In addition, a small peak at the void volume was consistently found. In some sera, a third high mol. wt peak reacting with anti-rat  $\beta_2$ m, located between the two others, was seen.

<sup>125</sup>I-rat  $\beta_2$ m was mixed with rat serum and incubated for 12 hr at 37°C. The mixture was then separated on the Sephadex G-200 column, and eluted fractions were subjected to  $\gamma$ -counting. Three peaks were found: at void volume, slightly after the last total protein peak, and at the place for free  $\beta_2$ m, in agreement with the profile found with radioimmunoassay on separated serum. The proportions between the two high mol. wt peaks, however, were different. The peak at void volume was twice as big as the other, and its proportion of total  $\beta_2$ m was doubled compared with the radioimmunoassay profile. Thus, the other high molecular weight peak was very much decreased, indicating that its  $\beta_2$ m content is only slowly exchanged. No fourth peak corresponding to that sometimes found with radioimmunoassay was seen.

On DEAE-cellulose, the serum  $\beta_2$ m separated into two peaks. The first was eluted in the position of free  $\beta_2$ m. The second contained the high mol. wt materials as shown by chromatography on Sephadex G-200.

A pattern with two peaks was also seen when serum was subjected to electrophoresis in agarose gel. The peaks migrated in the  $\gamma$ -globulin and in the slow  $\alpha_2$ -globulin regions, respectively.

#### DISCUSSION

Several findings indicate that the protein described

Table 3. Estimate of sequence difference (%) between different  $\beta_2$ ms from amino-acid compositions

| $\beta_2$ m-species | Rat | Human <sup>a</sup> | Rabbit <sup>b</sup> | Guinea Pig <sup>b</sup> | Mouse <sup>b</sup> | Dog <sup>c</sup> | Chicken <sup>d</sup> |
|---------------------|-----|--------------------|---------------------|-------------------------|--------------------|------------------|----------------------|
| Rat                 | —   | 33                 | 42                  | 41                      | 17                 | 30               | 60                   |
| Human               |     | —                  | 25                  | 14                      | 34                 | 33               | 51                   |
| Rabbit              |     |                    | —                   | 14                      | 51                 | 24               | 44                   |
| Guinea Pig          |     |                    |                     | —                       | 36                 | 26               | 42                   |
| Mouse               |     |                    |                     |                         | —                  | 31               | 41                   |
| Dog                 |     |                    |                     |                         |                    | —                | 19                   |
| Chicken             |     |                    |                     |                         |                    |                  | —                    |

<sup>a</sup>Data from complete amino-acid sequence (Peterson *et al.*, 1972).

<sup>b</sup>Calculated on the basis of 100 amino-acid residues per molecule. Rabbit  $\beta_2$ m (Berggård, 1974), Guinea pig  $\beta_2$ m (Cigén *et al.*, 1978) and Mouse  $\beta_2$ m (Natori *et al.*, 1975).

<sup>c</sup>Calculated on the basis of 100 amino-acid residues per molecule. Cys and Trp content assumed to be two of each (Poulik, 1973).

<sup>d</sup>Calculated on the basis of 100 amino-acid residues per molecule. Trp content assumed to be two (Winkler & Sanders, 1977).



Table 4. Cross-reactions between purified  $\beta_2$ ms as investigated by radio immunoassay. The 50% binding titre of the antisera are given

|                                        | $^{125}\text{I}$ rat $\beta_2\text{m}^a$ | $^{125}\text{I}$ guinea pig $\beta_2\text{m}^b$ | $^{125}\text{I}$ rabbit $\beta_2\text{m}^c$ | $^{125}\text{I}$ human $\beta_2\text{m}^d$ |
|----------------------------------------|------------------------------------------|-------------------------------------------------|---------------------------------------------|--------------------------------------------|
| *Goat anti-rat $\beta_2\text{m}$ 1*    | 1:5,000                                  | 1:4                                             | 1:1,000                                     | 1:100                                      |
| Goat anti-rat $\beta_2\text{m}$ 2      | 1:100,000                                | 1:200                                           | 1:10,000                                    | 1:10,000                                   |
| Goat anti-guinea pig $\beta_2\text{m}$ | 1:100                                    | 1:300,000                                       | 1:300                                       | 1:10                                       |
| Goat anti-rabbit $\beta_2\text{m}$     | 1:10,000                                 | 1:100                                           | 1:300,000                                   | 1:500                                      |
| Rabbit anti-human $\beta_2\text{m}$    | 1:30                                     | 1:20                                            | —                                           | 1:100,000                                  |

<sup>a</sup>  $^{125}\text{I}$  rat  $\beta_2\text{m}$  was used at 16 ng/l.<sup>b</sup>  $^{125}\text{I}$  guinea pig  $\beta_2\text{m}$  was used at 3.6 ng/l.<sup>c</sup>  $^{125}\text{I}$  rabbit  $\beta_2\text{m}$  was used at 6.0 ng/l.<sup>d</sup>  $^{125}\text{I}$  human  $\beta_2\text{m}$  was used at 2.0 ng/l.\*Goat anti-rat  $\beta_2\text{m}$  1 is from an early bleeding after one booster dose. Goat anti-rat  $\beta_2\text{m}$  2 is from a late bleeding after four booster doses.

in this paper is rat  $\beta_2\text{m}$ . Thus, the isolated protein is similar to  $\beta_2$ ms from other species with respect to mol. wt, antigenic structure, amino-acid composition, and the apparent presence of an analogous disulfide loop.

As starting material for purification of rat  $\beta_2\text{m}$ , we used concentrated urine from animals treated with sodium chromate. This treatment is known to lead to damage of the proximal tubules of the rat kidney, resulting in lysozymuria (Evan & Dail, 1974). The same type of starting material was used for preparation of  $\beta_2$ ms from rabbit (Berggård, 1974), guinea pig (Cigén *et al.*, 1978) and mice (B. Åkerström & I. Berggård, unpublished results).

Rat  $\beta_2\text{m}$  was relatively pure after the second purification step (DEAE-cellulose chromatography). The last two steps merely remove small quantities of contaminants. The final product was homogeneous on SDS-polyacrylamide gel electrophoresis. Besides the main component, small amounts of material with higher mobility towards the anode could be seen on polyacrylamide gel electrophoresis and on agarose gel

electrophoresis. The above-presented data indicate that this material is another form of rat  $\beta_2\text{m}$ . It is of interest that two forms of  $\beta_2\text{m}$  have previously been obtained also from human beings (Hall *et al.*, 1977) and guinea pigs (Cigén *et al.*, 1978).

The amino-acid composition of rat  $\beta_2$  has been compared with those of other  $\beta_2$ ms using a new index (Table 3). Values below sequence difference of 42% would be expected to occur only in one out of twenty comparisons of independently selected, unrelated proteins (Cornish-Bowden, 1977). The various homologues of  $\beta_2\text{m}$  have not been selected completely at random, and the degree of dependence is difficult to estimate. This limits the values of the index as evidence for relations between various  $\beta_2$ ms. With this reservation, the indexes given in Table 2 can be taken as evidence for a conservation of the  $\beta_2\text{m}$ -sequence. The relation between rat  $\beta_2\text{m}$  and  $\beta_2$ ms from other species was more evident on immunochemical comparisons. On Ouchterlony immunodiffusion analyses, rat  $\beta_2$  cross-reacted with  $\beta_2$ ms from four

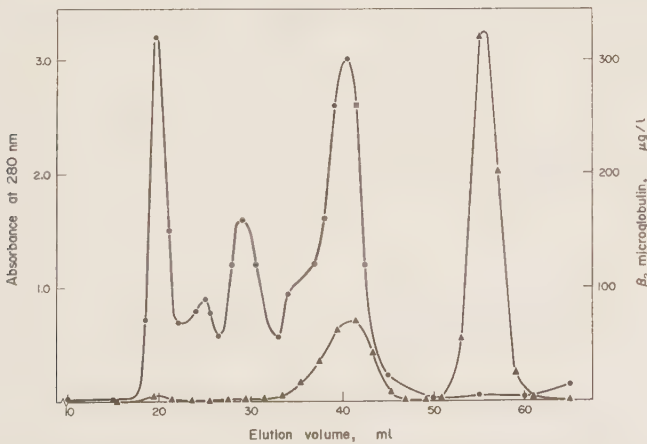


Fig. 7. Distribution in serum of rat  $\beta_2\text{m}$ . 1 ml of pooled serum from 100 rats was separated on a Sephadex G-200 column as described in Materials and Methods. Total protein was estimated by reading the absorbance at 280 nm (●—●). Rat  $\beta_2\text{m}$  was measured by radioimmunoassay (▲—▲).

other species (man, rabbit, guinea pig and mouse). The poor relation between the index and the presence of cross-reacting precipitating antibodies, which conclusively prove a relation, is exemplified by comparing index values (Table 3) for rat-rabbit and rat-guinea pig (clear cross-reactions) with the corresponding values for human-rabbit [weak cross-reaction (Berggård, 1974)] and human-guinea pig [no cross-reaction found (unpublished)]. On radioimmunoassay, we found different degrees of cross-reactivity in all investigated combinations. The radioimmunoassay results correlate poorly with both the results obtained on immunodiffusion analyses and the index values. This is exemplified by the weak radioimmunoassay titre for the goat anti-rat  $\beta_2$ m (second bleeding)- $^{125}$ I-guinea pig  $\beta_2$ m combination despite the clear precipitation line on Ouchterlony plates, and by strong titres in the rat-rabbit combinations despite a high index value. It is also interesting to compare the titres of the goat anti-rat  $\beta_2$ m bleedings. They differ with a factor of 20 in contrast to the titre difference on immunodiffusion analyses (factor of 4).

About 25% of serum  $\beta_2$ m, measured by radioimmunoassay, seemed to be in high mol. wt form. Most of this material was eluted just after the last major protein peak given by serum, which was separated by Sephadex G-200 chromatography. The material with high molecular weight might consist of  $\beta_2$ m-linked transplantation antigens (AgB) released from the cell surfaces. AgB-antigens isolated from Papain-digested liver cell membranes are known to contain a 12,000-dalton component, which cross-reacts with human  $\beta_2$ m (Katagiri *et al.*, 1975). The putative  $\beta_2$ m-AgB molecules in serum should be further studied with inbred rats with defined transplantation antigens. The  $\beta_2$ m peak at void volume of chromatography on Sephadex G-200 could consist of aggregated  $\beta_2$ m or of aggregated transplantation antigens. The former possibility is unlikely as the material in this peak is bound more firmly to the ion exchanger than free  $\beta_2$ m. Both materials discussed above could also be other kind of molecules complexed with  $\beta_2$ m. Both might have a low reactivity with antibodies directed against free  $\beta_2$ m and their quantities could be underestimated by the radioimmunoassay. Serum might also contain additional  $\beta_2$ m material, not measured here because of low reactivity with anti-rat  $\beta_2$ m and slow rate of  $\beta_2$ m exchange. High mol. wt  $\beta_2$ m peaks have previously been reported in sera from rabbits, dogs, mice and humans (Callahan *et al.*, 1976; Gordon & Kindt, 1976; Miyakawa *et al.*, 1973; Natori *et al.*, 1976).

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# HETEROLOGOUS INTERACTION BETWEEN $\beta_2$ -MICROGLOBULIN AND Ag-B ANTIGENS<sup>1</sup>

assical major histocompatibility antigens (HLA-A,B,C in H-2,D,K (L) in mouse, Ag-B in rat, etc.).—H antigens—to be critically involved as target molecules in the destruction of foreign or autologous cells by cytolytic T lymphocytes. The H antigens are integral membrane glycoproteins situated on most cell types. They are composed of two noncovalently associated polypeptide chains (2). The heavy chain (mol wt 44,000) carries the alloantigenic determinants, the carbohydrate moiety, and is responsible for the membrane penetration. The light chain (mol wt ~ 12,000) is a water-soluble protein ( $\beta_2$ -microglobulin ( $\beta_2$ M)) first isolated by Berggård (3) from pathological urine. Early suggestions that H antigens and immunoglobulins might share a common evolutionary origin have been substantiated by amino acid sequence analyses

TABLE 1. Binding of radiolabeled homologous and heterologous  $\beta_2$ M by purified Ag-B antigens<sup>a</sup>

| Step 1: incubation                                    | Step 2: Precipitating serum |            |            |
|-------------------------------------------------------|-----------------------------|------------|------------|
|                                                       | Normal BN                   | WF anti-BN | BN anti-WF |
| Experiment A                                          |                             |            |            |
| Rat $\beta_2$ M + Ag-B + 0                            | 739                         | 778        | 2,406      |
| Rat $\beta_2$ M + Ag-B + Rat $\beta_2$ M <sup>b</sup> | 808                         | 864        | 745        |
| Rat $\beta_2$ M + Ag-B + Hum $\beta_2$ M              | 772                         | 750        | 792        |
| Rat $\beta_2$ M + Ag-B + Rab $\beta_2$ M              | 795                         | 774        | 744        |
| Rat $\beta_2$ M + Ag-B + GP $\beta_2$ M               | 760                         | 855        | 776        |
| Rat $\beta_2$ M + Ag-B + Hum $\alpha_1$ M             | 824                         | 766        | 2,423      |
| Rat $\beta_2$ M + 0 + 0                               | 766                         | 824        | 812        |
| Experiment B                                          |                             |            |            |
| Hum $\beta_2$ M + Ag-B + 0                            | 2,238                       | 2,465      | 16,022     |
| Hum $\beta_2$ M + Ag-B + Rat $\beta_2$ M              | 2,644                       | 2,652      | 2,759      |
| Hum $\beta_2$ M + Ag-B + Hum $\beta_2$ M              | 2,877                       | 2,680      | 2,741      |
| Hum $\beta_2$ M + Ag-B + Rab $\beta_2$ M              | 2,749                       | 2,892      | 2,794      |
| Hum $\beta_2$ M + Ag-B + GP $\beta_2$ M               | 2,620                       | 2,566      | 2,848      |
| Hum $\beta_2$ M + Ag-B + Hum $\alpha_1$ M             | 2,593                       | 2,660      | 14,870     |
| Hum $\beta_2$ M + 0 + 0                               | 3,026                       | 3,030      | 3,108      |
| Experiment C                                          |                             |            |            |
| Rab $\beta_2$ M + Ag-B + 0                            | 1,878                       | 2,186      | 6,158      |
| Rab $\beta_2$ M + Ag-B + Rat $\beta_2$ M              | 2,158                       | 2,142      | 1,637      |
| Rab $\beta_2$ M + Ag-B + Hum $\beta_2$ M              | 2,044                       | 2,324      | 2,281      |
| Rab $\beta_2$ M + Ag-B + Rab $\beta_2$ M              | 1,856                       | 2,058      | 2,038      |
| Rab $\beta_2$ M + Ag-B + GP $\beta_2$ M               | 1,854                       | 1,968      | 1,798      |
| Rab $\beta_2$ M + Ag-B + Hum $\alpha_1$ M             | 2,200                       | 2,254      | 5,788      |
| Rab $\beta_2$ M + 0 + 0                               | 2,026                       | 2,238      | 2,278      |
| Experiment D                                          |                             |            |            |
| GP $\beta_2$ M + Ag-B + 0                             | 2,925                       | 2,834      | 9,976      |
| GP $\beta_2$ M + Ag-B + Rat $\beta_2$ M               | 3,143                       | 3,036      | 3,179      |
| GP $\beta_2$ M + Ag-B + Hum $\beta_2$ M               | 3,120                       | 3,107      | 2,764      |
| GP $\beta_2$ M + Ag-B + Rab $\beta_2$ M               | 3,039                       | 2,850      | 2,909      |
| GP $\beta_2$ M + Ag-B + GP $\beta_2$ M                | 2,822                       | 2,704      | 2,867      |
| GP $\beta_2$ M + Ag-B + Hum $\alpha_1$ M              | 3,216                       | 2,922      | 9,547      |
| GP $\beta_2$ M + 0 + 0                                | 3,002                       | 2,846      | 2,950      |
| Experiment E                                          |                             |            |            |
| Hum $\alpha_1$ M + Ag-B + 0                           | 483                         | 462        | 490        |
| Hum $\alpha_1$ M + 0 + 0                              | 540                         | 529        | 508        |

Results are mean cpm values for two aliquots. An estimation of the technical error based on variation within duplicates from several experiments, gave a SD of 4.9% (269 df). Total cpm for the aliquots were 22,000 in experiment A, 19,000 in experiment B, 59,000 in experiment C, 61,000 in experiment D, and 49,000 in experiment E.

<sup>a</sup> In some incubations, unlabeled proteins,  $\beta_2$ Ms or  $\alpha_1$ M at 10  $\mu$ g/ml, were added. Hum, human; Rab, rabbit; GP, guinea pig.

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TABLE 2. Inhibition of interaction between human  $\beta_2$ M and Ag-B antigens by heterologous sera<sup>a</sup>

| Step 1: incubation                                                    | Step 2: precipitating serum |            |                                        |     |
|-----------------------------------------------------------------------|-----------------------------|------------|----------------------------------------|-----|
|                                                                       | Normal BN                   | BN anti-WF | Specific precipitation ( $\Delta$ cpm) | (%) |
| Experiment A                                                          |                             |            |                                        |     |
| <sup>125</sup> I-Hum $\beta_2$ M + Ag-B + 0                           | 2,868 <sup>b</sup>          | 15,562     | 12,694                                 | 100 |
| <sup>125</sup> I-Hum $\beta_2$ M + Ag-B + cow serum (1:10)            | 4,386                       | 4,476      | 90                                     | 0.7 |
| <sup>125</sup> I-Hum $\beta_2$ M + Ag-B + rhesus monkey serum (1:10)  | 3,599                       | 3,794      | 195                                    | 1.5 |
| <sup>125</sup> I-Hum $\beta_2$ M + Ag-B + mouse serum (1:10)          | 4,935                       | 5,144      | 209                                    | 1.6 |
| Experiment B                                                          |                             |            |                                        |     |
| <sup>125</sup> I-Hum $\beta_2$ M + Ag-B + 0                           | 2,496                       | 14,850     | 12,354                                 | 100 |
| <sup>125</sup> I-Hum $\beta_2$ M + Ag-B + spiny dogfish serum (1:10)  | 3,731                       | 15,848     | 12,117                                 | 98  |
| <sup>125</sup> I-Hum $\beta_2$ M + Ag-B + G-200 III $\times$ 8 (1:10) | 3,414                       | 12,274     | 8,860                                  | 72  |

<sup>a</sup> In experiment A, sera from cow, rhesus monkey, or mouse were included in the incubation mixtures. In experiment B, incubation mixtures contained serum or a concentrated pool (G-200 III) of Sephadex G-200 chromatographed serum from spiny dogfish (*S. acanthias*). The pool corresponded to  $K_{av}$  values 0.10 to 0.25 and was concentrated to a volume 1/8 of the separated serum before use. The other pools of the separated fish serum did not markedly block the specific precipitation (<10% inhibition).

<sup>b</sup> Results are mean cpm values for two aliquots. Technical error  $\approx$  4.9% (see Table 1).

of  $\beta_2$ M (6, 7) and, more recently, of the H antigen heavy chain (8, 9). Accordingly,  $\beta_2$ M and the H antigen heavy chain have been well conserved through evolution, as shown by sequence studies and immunological cross-reactions in various mammalian species and chicken (10–12). The present work demonstrates this evolutionary conservation also by heterologous interactions between  $\beta_2$ M and the H antigen heavy chain. The results suggest that these types of interactions could be a tool in pursuing H antigen analogues in lower species.

Binding of radiolabeled  $\beta_2$ M to purified Ag-B antigens was carried out as follows: Step 1: Purified papain-solubilized splenocyte Ag-B antigens (~5 ng/ml; estimated from  $\beta_2$ M content) from inbred Wistar-Furth (WF) rats (L. Björck, B. Axelsson, and L. Löfdberg, unpublished data) and radiolabeled  $\beta_2$ M (2 to 10 ng/ml) from rat (12), man (13), rabbit (14), or guinea pig (15) were mixed and incubated at 37 C for 24 hr. This procedure has been shown to allow binding of radiolabeled rat  $\beta_2$ M to high molecular weight complexes in rat serum (12), and also binding of  $\beta_2$ M to high molecular weight complexes in heterologous sera (L. Löfdberg, R. Cigén, and L. Björck, unpublished observations). Step 2: Antiserum specific for WF alloantigens, raised in inbred Brown Norway (BN) rats (BN anti-WF) or control sera (WF anti-BN or normal BN), were added (25  $\mu$ l) to aliquots (200  $\mu$ l) of the reaction mixtures. Incubation was proceeded at room temperature for at least 5 hr. Step 3: Immune complexes were separated from unbound  $\beta_2$ M by polyethylene-glycol exclusion (16) and the radioactivity in the precipitate was determined.

Binding of radiolabeled rat  $\beta_2$ M to Ag-B antigens was demonstrated as specific precipitation of radioactivity by alloantisera directed against the relevant Ag-B antigens, which was lost if the Ag-B antigens were excluded from step 1 (Table 1, A). The specificity of the binding was confirmed as it could be completely blocked by unlabeled rat  $\beta_2$ M but not by the pre-



sumably irrelevant protein human  $\alpha_1$ -microglobulin ( $\alpha_1$ M). This suggests that radiolabeled rat  $\beta_2$ M is exchanged with the light chain of the Ag-B antigens. Recent studies in the human system (17) support this conclusion. It was now very interesting to find that this binding could also be completely blocked by  $\beta_2$ M from three other species (Table 1, A). These findings are interpreted as an evolutionary conservatism in the  $\beta_2$ M-binding site on the H antigen heavy chain and the corresponding determinants on  $\beta_2$ M. Moreover, the Ag-B antigens could also specifically bind radiolabeled  $\beta_2$ M from man, rabbit, or guinea pig (Table 1, B to D). These heterologous bindings of  $\beta_2$ M could always be completely blocked by unlabeled  $\beta_2$ M from the four species, but never by human  $\alpha_1$ M. Predictably, Ag-B antigens could not bind radiolabeled human  $\alpha_1$ M (Table 1, E). These results corroborate and extend recent observations by Hester et al. (18), which suggested that H 2 antigens on mouse lymphocytes could act as a receptor for human  $\beta_2$ M.

$\beta_2$ M seems to influence the conformation of H antigens (19). Thus, the exchange of homologous for heterologous  $\beta_2$ M in the H antigen molecule might lead to altered conformation; however, no effect on allospecificity is apparent, as alloantisera were used in this study.

The heterologous binding of  $\beta_2$ M by H antigens could be expected to be useful for detection and purification of analogous molecules in species where these reagents are not available. Sera from cow, rhesus monkey, and mouse abrogated the binding of radiolabeled human  $\beta_2$ M to Ag-B antigens (Table 2, A), supporting this assumption. These sera did not interfere with the binding of radiolabeled Ag-B antigens by BN anti-WF alloantibodies (not shown). Thus, their inhibitory activity is specifically exerted on the heterologous Ag-B  $\beta_2$ M interaction.

Evolutionary studies on  $\beta_2$ M and H antigens should have strong implications on our understanding of the evolution of the immune system. However, unambiguous biochemical demonstration of these molecules has, as far as we know, only been achieved in mammals and birds. Therefore, it is very promising to find that a concentrated pool of Sephadex G-200-separated serum from spiny dogfish (*Squalus acanthius*) can partially inhibit the Ag-B-binding of radiolabeled human  $\beta_2$ M (Table 2, B). Such inhibition is also executed by a similar concentrated pool of gel chromatographed serum from an aglomerular fish (*Lophius americanus*) (specific precipitation, 65%). Gordon and Kindt (20) previously found heterologous  $\beta_2$ M radioimmunoassay inhibitory activity in sera from lower vertebrates. In our hands, however, such activity is restricted to the void fraction after Sephadex G-200 chromatography. This is in contrast to

the molecules responsible for the inhibition of the Ag-B-<sup>125</sup>I-labeled human  $\beta_2$ M interaction, which, both in *S. acanthius* and *L. americanus*, are included in the separation range of Sephadex G-200.

In summary, we have demonstrated a heterologous interaction between H antigens and  $\beta_2$ M that promises to be a valuable tool for biochemical studies of H antigens in lower species.

*Note added in proof.* Since the submission of this manuscript we have found that purified papain-solubilized Ag-B antigens from inbred BN rats show the same kind of reactivity with heterologous  $\beta_2$ M as Ag-B antigens from WF rats.

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## ON THE INTERACTION BETWEEN $\beta_2$ -MICROGLOBULIN AND THE HEAVY CHAIN OF MHC CLASS 1 ANTIGENS

by

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## ABSTRACT

The heterologous interaction between  $\beta_2$ -microglobulin ( $\beta_2m$ ) and rat MHC antigens (AgB) was measured in a two-step binding assay. 1) Binding of radiolabelled  $\beta_2m$  to AgB antigens. 2) Immuno-precipitation of  $\beta_2m$ -AgB-complexes with AgB-antisera. The effects of varying the concentrations of the three reactants involved were studied. The molecular events taking place in the two steps were analyzed by gel chromatography. The  $\beta_2m$ -AgB-complex had the apparent size of albumin, and reacted completely with specific alloantisera. AgB antigens prepared from Wistar/Furth (AgB 2) and Brown Norway (AgB 3), respectively, both effectively bound heterologous  $\beta_2m$ . The times for association and dissociation, respectively, at 37°C, were of the same order, but dissociation slightly slower. Association was markedly temperature-dependent and was considerably slower at low temperatures. All these processes were slower for AgB 3 than for AgB 2 antigens. The association constant for the interaction between AgB 2 antigens and  $^{125}I$ -human  $\beta_2m$  was estimated by Scatchard analyses to be about  $3 \times 10^9 M^{-1}$ . Contribution to the heterologous interaction by products from various rat MHC subloci (A, B, and C) was investigated by the introduction of sublocus-specific antisera in Step 2. The reaction apparently involved neither class 2 antigens (sublocus B), nor the presumed rat Qa-homologue (sublocus C). Classical class 1 antigens (sublocus A) clearly contributed to the binding. However, a monoclonal antibody against products from rat MHC class 1 genes only precipitated less than half of the AgB-complexed  $\beta_2m$ . Thus, at least two AgB 2 alloantigen molecules seem to participate in the reaction. This, in turn, indicates that the rat genome may contain multiple class 1 genes.

## INTRODUCTION

$\beta_2$ -Microglobulin ( $\beta_2m$ ) is a low-molecular-weight protein detected in biological fluids and on most nucleated cell types. It is apparently a member of a protein superfamily, that also includes immunoglobulins, MHC class 1- and 2 antigens, and Thy-1-antigen (cf. Robertson, 1982 and Williams & Gagnon, 1982). MHC class 1 antigens are expressed on the cell surface in physical association with  $\beta_2m$  (cf. Berggård *et al.*, 1980). Also, several workers report  $\beta_2m$  to be associated with a number of other molecules (cf. Berggård *et al.*, 1980), some of which will probably turn out to be additional members of the above-mentioned protein family. Thus, evolutionary studies on  $\beta_2m$  and  $\beta_2m$ -binding molecules may shed light on the origin and function of this protein class. Considering this background we have examined the evolutionary conservation of the interaction between  $\beta_2m$  and  $\beta_2m$ -binding molecules in various test models (Lögberg *et al.*, 1980 and 1981 a,b). In a preliminary report (Lögberg *et al.*, 1980), we demonstrated that rat MHC antigens (AgB = RT1) can bind not only rat  $\beta_2m$ , but also  $\beta_2m$ -homologues from other species. Subsequently, other groups reported similar findings with MHC antigens from mouse (Schmidt *et al.*, 1981) and man (Church *et al.*, 1982). In the present paper, we continue our investigation of this kind of heterologous interaction.

## MATERIALS AND METHODS

### $\beta_2$ -Microglobulins.

Human (Berggård & Bearn, 1968), rat (Lögdberg *et al.*, 1979), and rabbit (Björck & Berggård, 1981)  $\beta_2$ ms were all purified in our own laboratory. Antisera against these proteins are described in the respective papers.

### Rats and spleens.

Breeding pairs of the inbred rat strains Wistar/Furth (WF; AgB 2 = RT<sup>1u</sup>) and Brown Norway (BN; AgB 3 = RT<sup>1n</sup>) were a kind gift of Professor Hans Olov Sjögren, University of Lund, Sweden. They were maintained in strict brother-sister mating, and served as a source of spleens for AgB antigen purification. They were also used as donors and recipients in the production of alloantisera. Rat spleens from these strains were also provided by Professor Sjögren.

### Rat MHC class 1 antigens.

AgB antigens were purified (Björck *et al.*, unpublished) from membrane preparations of rat spleens. Briefly, rat spleens were homogenized and then subjected to ultracentrifugation. The 105,000 x g pellet of the 10,000 x g supernatant was papain-digested. After repeated ultracentrifugation, the 105,000 x g supernatant was fractionated by ion-exchange chromatography on DEAE-cellulose (Serva Feinbiochemia, Heidelberg, West-Germany), affinity chromatography on *Lens culinaris* lectin-Sepharose (Pharmacia, Uppsala, Sweden), and gel chromatography on Sephadex G-200 (Pharmacia), in that order. AgB antigens, detected by rat  $\beta_2$ m-radioimmunoassay (RIA) (Lögdberg *et al.*, 1979), emerged from the Sephadex G-200-column as a symmetrical peak with the apparent size of human albumin. When <sup>125</sup>I-radiolabelled and examined by SDS-polyacrylamid gel electrophoresis, the resulting AgB-preparation consisted mainly of the expected 37,000 and 12,000 dalton components. The AgB class 1 antigen quantities were estimated from the RIA-determined  $\beta_2$ m-content, assuming a 1:1 relation between the light and heavy chains.

### AgB-antisera

We produced alloantisera (BN anti-WF and WF anti-BN, respectively) by repeated intraperitoneal injections of spleen cell suspensions, containing  $50 \times 10^6$  leukocytes. Animals were immunized and then boosted three times with intervals of two weeks. One week after the last booster, the rats were bled. We also received alloantisera produced in a similar way as a gift from Professor Sjögren. Dr. G. W. Butcher, Cambridge, England, kindly gave us a monoclonal antibody (NR3/31), reactive against the AgB-A products of WF-rats (DA anti-AO) (Howard *et al.*, 1979). We used this reagent from a stock solution of partly purified IgG (6 mg protein/ml). Finally, Dr. E. Günther, Freiburg, West Germany, generously donated a set of alloantisera reactive against products from various subloci of the WF-MHC. These antisera are: 93-6 (LEW.AR2 x LEW.1N)F<sub>1</sub> anti-(LEW.1AR1 x LEW.1N)F<sub>1</sub>, that reacts with AgB-B (Ia-) alloantigens, 151-62 (LEW.1WR1 x LEW.1N)F<sub>1</sub> anti-LEW.1W, that recognize products from the AgB-C sublocus, and 145 (LEW.1A anti-LEW.1W), which theoretically could react with AgB-A, B, and C antigens (cf. Stock & Günther, 1982).

### Preparation of $^{125}\text{I}$ -radiolabelled $\beta_2$ -microglobulin.

$\beta_2\text{m}$  was radiolabelled by the chloramin T-method (Greenwood et al., 1963). The radiolabelled  $\beta_2\text{m}$  was separated by gel chromatography on columns of Sephadex G-25 or G-50 (Pharmacia), in order to remove free iodine.

### Assay for binding of $^{125}\text{I}$ - $\beta_2$ -microglobulin to AgB antigens.

Step 1. Mixtures of purified AgB antigens and  $^{125}\text{I}$ - $\beta_2\text{m}$  were incubated in 0.1 M phosphate buffer, pH 7.4, + 0.1 % bovine serum albumin + 0.02 %  $\text{NaN}_3$ . Step 2. Antiserum against AgB or control serum, respectively, was added to aliquots of the incubation mixture. After a second incubation, antigen-antibody-complexes were precipitated by addition of 12.5 % bovine serum (carrier) and polyethylenglycol 6000 (PEG; Kebo, Malmö, Sweden), followed by centrifugation. The pellets were subjected to  $\gamma$ -counting, after removal of the supernatant. Technical error (variation between duplicates) was  $\sim 5$  %.

### Determination of binding constant.

The same amounts of WF-AgB antigens were incubated with various concentrations of radiolabelled human  $\beta_2\text{m}$ . After precipitation of bound  $\beta_2\text{m}$  and correction for the fact that part of the radiolabelled  $\beta_2\text{m}$  appeared inaccessible to the AgB antigens, the radioactive counts were used to calculate the concentrations of free and bound  $\beta_2\text{m}$ . Such data were used for Scatchard-analyses (Scatchard, 1949).

### Gel chromatography.

In some experiments, incubation mixtures were applied to a column of Sephadex G-200 superfine (Pharmacia; 1 x 100 cm), equilibrated with 0.02 M Tris-HCl, pH 8.0, + 0.15 M NaCl + 0.02 %  $\text{NaN}_3$ . Flow rate: 1.5 ml/h. Fractions were analyzed for  $\beta_2\text{m}$  by  $\gamma$ -counting.

## RESULTS

### Detection of the heterologous interaction between $\beta_2$ -microglobulin and AgB antigens - influence of $\beta_2$ -microglobulin, AgB antigen, and alloantiserum concentrations. Binding constant.

After incubation of  $^{125}\text{I}$ - $\beta_2\text{m}$  with AgB antigens at  $37^\circ\text{C}$ , AgB-complexed  $\beta_2\text{m}$  could be detected in a second step by immunoprecipitation with specific alloantisera (Lögberg et al., 1980). Fig. 1 demonstrates the effect of different alloantiserum concentrations in Step 2. The Figure contains data from an experiment with AgB 2 antigens, but similar data were also obtained with AgB 3 antigens, although higher concentrations of allo-antiserum had to be used in the latter experiments.



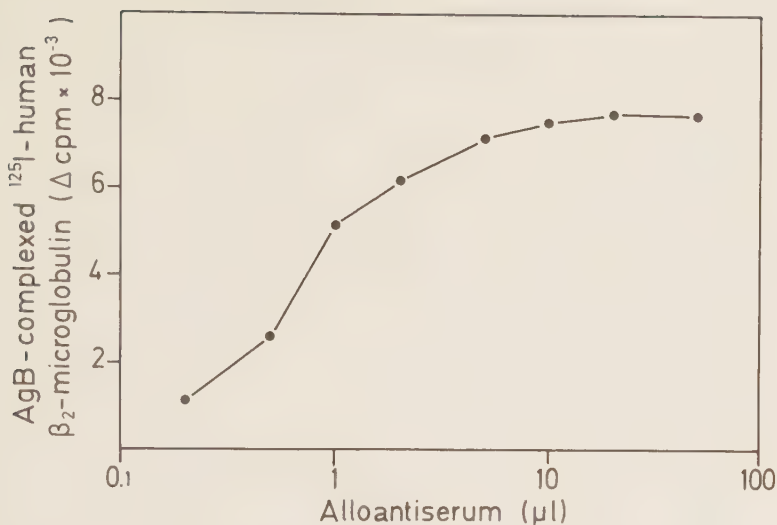


Figure 1

$^{125}\text{I}$ -human  $\beta_2\text{m}$  (40 ng/ml  $\sim 5 \times 10^5$  cpm/ml) and 20 ng of AgB 2 antigens/ml were incubated overnight at  $37^\circ\text{C}$ . The incubation mixtures were then divided into 200  $\mu\text{l}$  aliquots, to each of which were added 50  $\mu\text{l}$  of precipitating serum, i.e., various dilutions of BN anti-WF in normal BN serum. After 5 h of incubation at room temperature, immune complexes were PEG-precipitated and counted. Background precipitation with the normal BN serum was subtracted and results are given as specific precipitation ( $\Delta \text{cpm}$ ).

The AgB 2 antigen-binding capacity of a constant amount of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  approached saturation at high concentrations of AgB 2 antigens (not shown). However, the reaction could not be driven to complete saturation, as this would involve unacceptably high concentrations of alloantiserum in Step 2. The data indicated that only a fraction ( $\sim 20\%$ ) of the  $^{125}\text{I}$ - $\beta_2\text{m}$  was accessible for complex-formation with AgB 2 antigens.

The  $\beta_2\text{m}$ -binding capacity of a constant amount of AgB 2 antigens could be saturated by high concentrations of  $^{125}\text{I}$ -human  $\beta_2\text{m}$ . At saturation, the approximate quantitative composition of the  $\beta_2\text{m}$ -AgB 2-complexes was 1:2.5 (same f.l. t.:v different AgB 2 antigen preparations). With low concentrations of  $\beta_2\text{m}$ , the bound fraction of  $\beta_2\text{m}$  again approached 20 - 30 % (varied between different batches of radiolabelled  $\beta_2\text{m}$ ). Thus, after correction for the inaccessible fraction of  $^{125}\text{I}$ -human  $\beta_2\text{m}$ , the data were plotted according to Scatchard (1949). From such analyses, a binding constant of  $3 \times 10^9 \text{ M}^{-1}$  could be calculated (mean of several determinations on two AgB 2 antigen preparations). Fig. 2 shows the results from one such experiment. The binding of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  to AgB 2 antigens

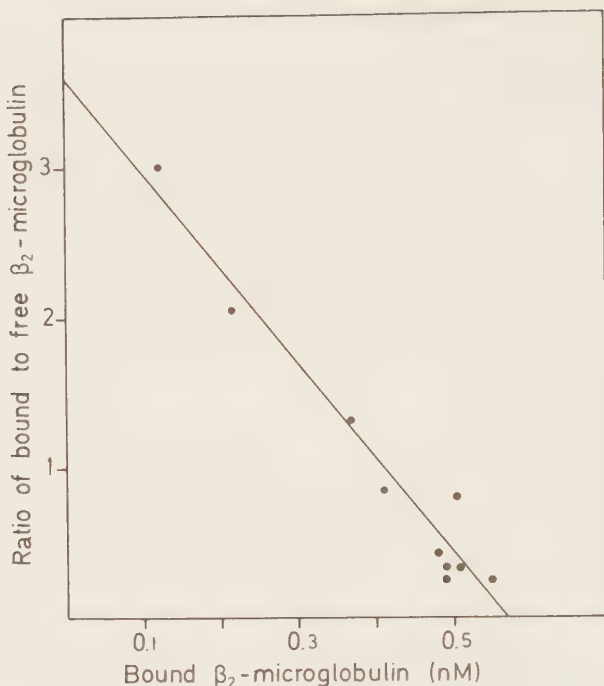


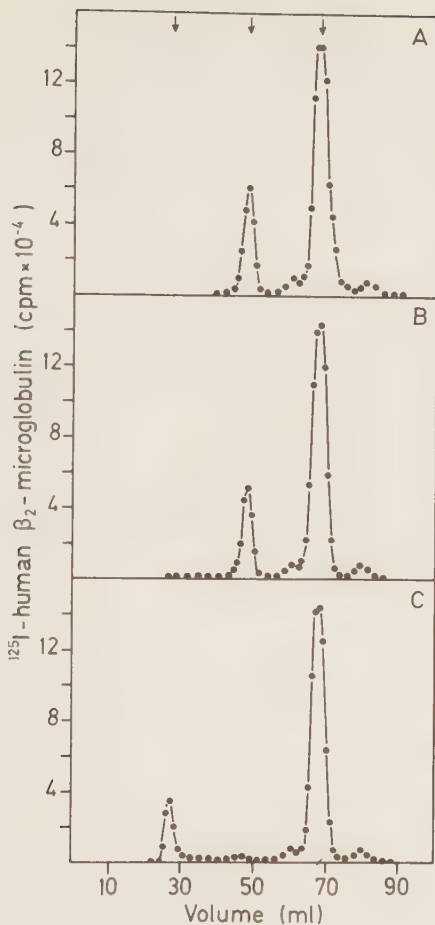
Figure 2

AgB 2 antigens (20 ng/ml) were incubated overnight with various amounts of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  ( $10^6$  cpm  $\sim 80$  ng) at  $37^\circ\text{C}$ .  $\beta_2\text{m}$ -AgB-complexes were then precipitated from 200  $\mu\text{l}$  aliquots with 20  $\mu\text{l}$  BN anti-WF or with normal BN serum (control). From the specific precipitations, the concentrations of bound and free  $\beta_2\text{m}$ , respectively, were calculated. These data were used for Scatchard-analysis (Scatchard, 1949). Thus, the ratio of bound to free  $\beta_2\text{m}$  (B/F) was plotted against the concentration of bound  $\beta_2\text{m}$  (B). Using the following expression for the equilibrium equation:  $B/F = K(A_0 - B)$ , where  $A_0$  is the concentration of binding sites for  $\beta_2\text{m}$  and  $K$  is the binding constant,  $A_0$  is derived from the intercept with the abscissa.  $K$  can now be calculated from the intercept with the ordinata. In this particular experiment, 0.57 nM of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  was bound at saturation, which gave a binding constant of  $6.3 \times 10^9 \text{ M}^{-1}$ .

could be inhibited by addition of unlabelled  $\beta_2\text{m}$  to the Step 1-incubations. The same concentrations of human, rat, or rabbit  $\beta_2\text{m}$ , respectively, gave the same degrees of inhibition (not shown), indicating similar binding constants for these proteins.

Alloantiserum can completely precipitate the  $\beta_2$ -microglobulin-AgB-antigen-complexes.

Fig. 3 shows the Sephadex-G-200 gel chromatograms of the incubation mixtures from different steps of the binding assay. The  $\beta_2\text{m}$ -AgB 2-complexes formed in Step 1, emerged from the Sephadex G-200-column as a symmetrical peak with the same elution volume as human albumin (Fig. 3A).



**Figure 3**

AgB 2 antigens (100 ng) and 80 ng of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  ( $\sim 10^6$  cpm) were incubated overnight in a total volume of 1 ml at  $37^\circ\text{C}$ . The size-distribution of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  was analyzed by Sephadex G-200 superfine gel chromatography, either directly (A), or after addition of 100  $\mu\text{l}$  of normal BN serum (B) or BN anti-WF (C), and incubation at room temperature for 5 h. Arrows indicate the elution volumes of blue dextran ( $V_0$ ), human albumin, and human  $\beta_2\text{m}$ , respectively.

Incubation with normal BN serum in Step 2 did not alter the subsequent  $\beta_2\text{m}$ -distribution in the gel chromatogram (Fig. 3B), whereas after incubation with BN anti-WF, complexed  $\beta_2\text{m}$  appeared in the void volume alone (Fig. 3C). Thus, the BN anti-WF apparently can react with all the  $\beta_2\text{m}$ -binding molecules in the AgB 2 antigen preparation, leading to formation of large immune-complexes. Experiments with AgB 3 antigens gave similar results.

Influence of time and temperature on the heterologous association of  $\beta_2$ -microglobulin to AgB antigens. Dissociation.

The binding of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  to AgB 2 or AgB 3 antigens, respectively, was markedly temperature-dependent (Fig. 4). At  $37^\circ\text{C}$ , the binding to

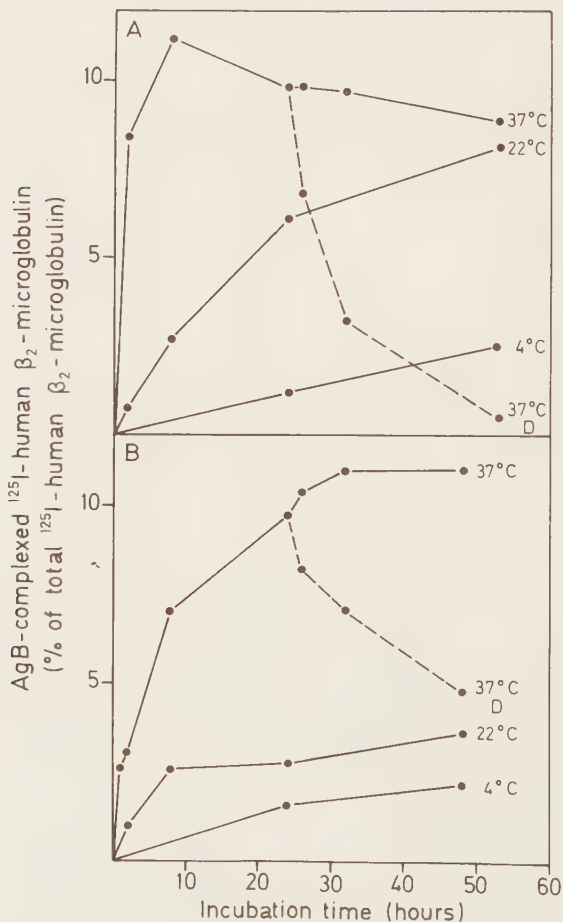


Figure 4

$^{125}\text{I}$ -Human  $\beta_2\text{m}$  (40 ng/ml  $\sim 5 \times 10^5$  cpm/ml) was incubated with 20 ng/ml of AgB 2 antigens (A) or 60 ng/ml of AgB 3 antigens (B), at various temperatures for varying lengths of time. To some incubations, 4  $\mu\text{g}/\text{ml}$  of unlabelled human  $\beta_2\text{m}$  were added after 24 h of incubation ( $37^\circ\text{C}$  D). The amounts of  $\beta_2\text{m}$ -AgB-complexes were determined as specific precipitation from 200  $\mu\text{l}$  aliquots with 20  $\mu\text{l}$  of the relevant alloantiserum: BN anti-WF (A) or WF anti-BN (B).

AgB 2 antigens reached saturation within 12 h (Fig. 4A), whereas more than 24 h was required to saturate the AgB 3 antigens (Fig. 4B). Association was much slower at room temperature, especially for AgB 2 antigens. At 4°C, less than 30% of maximum association had occurred after 2 days.

Already complexed  $^{125}\text{I}$ - $\beta_2\text{m}$  could be completely dissociated by the addition of excess amounts of unlabelled  $\beta_2\text{m}$  (Fig. 4A). Dissociation was somewhat slower than association.

#### Influence of salt concentration.

The heterologous interaction between  $^{125}\text{I}$ - $\beta_2\text{m}$  and AgB 2 antigens was not affected by NaCl even at high concentrations (3M), whereas 1 M of the chaotropic salt NaSCN abrogated the binding.

Participation of products from rat MHC subloci in the heterologous interaction between  $\beta_2\text{m}$  and AgB antigens suggests more than one type of  $\beta_2\text{m}$ -binding molecule.

Table 1 demonstrates that antisera against AgB-B2 and -C2-products did not precipitate the  $\beta_2\text{m}$ -AgB2-complexes. An anti-AgB-A2, B2, C2 serum, however, gave a significant reaction. A monoclonal antibody against rat AgB2 class 1 antigens could only precipitate about half of the  $\beta_2\text{m}$ -AgB-complexes (Fig. 5)

Table 1

Rat MHC sublocus-analysis of the  $\beta_2$ -microglobulin-binding AgB-antigens<sup>a</sup>.

| Step 1                         | Step 2                                | cpm                  |
|--------------------------------|---------------------------------------|----------------------|
| Incubation                     | Precipitating serum                   | (mean of duplicates) |
| 20 ng AgB 2 +                  | 20 $\mu\text{l}$ Normal BN            | 4549                 |
| 7 ng $^{125}\text{I}$ -human   | 20 $\mu\text{l}$ BN anti-WF           | 13496                |
| $\beta_2\text{m}$ (82,242 cpm) | 100 $\mu\text{l}$ Normal Lewis        | 4307                 |
| in 200 $\mu\text{l}$ buffer    | 100 $\mu\text{l}$ 145 (anti-A2,B2,C2) | 7472                 |
|                                | 100 $\mu\text{l}$ 93-6 (anti-B2)      | 5006                 |
|                                | 100 $\mu\text{l}$ 151-62 (anti-C2)    | 5174                 |

<sup>a</sup>Incubation overnight at 37°C followed by addition of precipitating serum and five hours of incubation at room temperature.



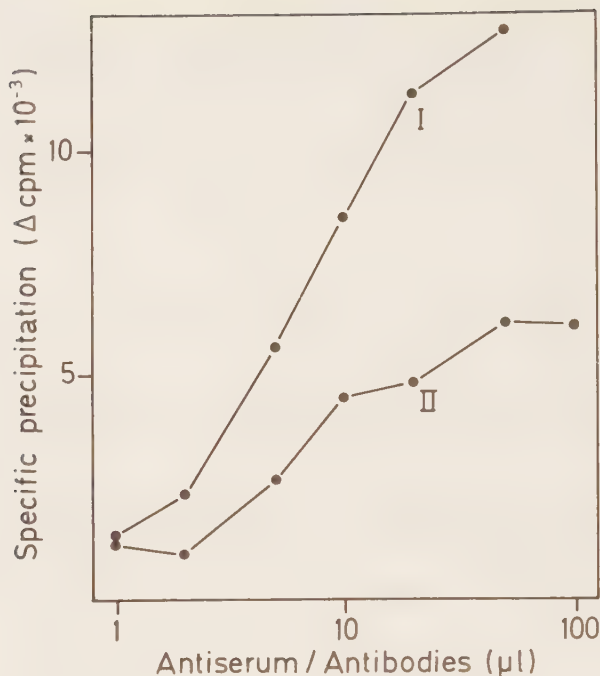


Figure 5

The amounts of  $\beta_2m$ -AgB 2-complexes that could be precipitated from 200  $\mu l$ -aliquots of incubations of AgB 2 (100 ng/ml) and  $^{125}I$ -human  $\beta_2m$  (40 ng/ml  $\sim 5 \times 10^5$  cpm/ml), with various concentrations of two different anti-AgB 2 (BN anti-WF or NR3/31), were determined as described in the legend for Fig. 1.

## DISCUSSION

The fact that the light chain ( $\beta_2m$ ) of the MHC class 1 antigens can be exchanged with exogenously-added  $\beta_2m$  has only recently been realized (Graf *et al.*, 1982; Hyafil *et al.*, 1979). Apparently, even heterologous  $\beta_2m$  can replace the homologous counterpart in the MHC antigens in such an exchange reaction (Church *et al.*, 1982; Lögdberg *et al.*, 1980; Schmidt *et al.*, 1981). This report describes some characteristics of the heterologous interaction between  $\beta_2m$  and AgB antigens. The concentrations of each of the three reactants ( $\beta_2m$ , AgB, and alloantiserum) were expectedly of critical importance for the results. Fig. 1 demonstrates the necessity to use excess concentrations of alloantiserum in Step 2. The saturation of AgB antigens with  $^{125}I$ -human  $\beta_2m$  indicates that a little more than one  $\beta_2m$ -molecule bound to each AgB-molecule (w/w-relation 1:2.5 instead of the expected 1:4). The uncertainty in the determination of the AgB antigen and  $\beta_2m$  concentrations is probably a sufficient explanation for the divergence from a 1:1 (mol/mol)-relation. However, presence in the AgB-antigen

preparations of free class 1 antigen heavy chains reactive with  $\beta_2m$  could also explain this result. Participation of free  $\beta_2m$ -binding sites would be compatible with the observation that dissociation was somewhat slower than association. On the other hand, free heavy chains are claimed to undergo conformational changes leading to decreased  $\beta_2m$ -binding capacity and lost alloantigenicity (Lancet et al., 1979; Vincent & Revillard, 1979).

The radiolabelling may specifically alter the AgB-binding site on  $\beta_2m$ , as only a fraction of the radiolabelled  $\beta_2m$  appeared to be accessible for binding to the AgB antigens. This may indicate direct involvement of tyrosin-residues at this binding site.

There are data supporting the presence in serum and on splenocytes, respectively, of  $\beta_2m$ -binding molecules that do not contain alloantigenic determinants (Kvist & Peterson, 1978; Natori et al., 1976; Sege et al., 1979; Sege & Peterson, 1980). Thus, when we were not able to precipitate more than 80 - 90 % of gel chromatography-purified  $\beta_2m$ -AgB-complexes (Fig. 3A) with alloantiserum, but anti- $\beta_2m$  could precipitate 100 %, such molecules were implicated. However, gel chromatography after Step 2 revealed translocation of AgB- $\beta_2m$ -complexes to the void volume, without residual complexes of albumin-size (Fig. 3C). This perhaps suggests that there is some dissociation of the AgB- $\beta_2m$ -complexes during Step 2.

It is interesting to note the different properties of AgB 2 and AgB 3 antigens (Fig. 4). Such differences between alleles could perhaps explain many of the divergent results regarding the interactions between  $\beta_2m$  and  $\beta_2m$ -binding molecules (Anderson et al., 1975; Church et al., 1982; Hester et al., 1979; Hyafil et al., 1979; Lögdberg et al., 1980 and 1981 a,b; Schmidt et al., 1981; Sege et al., 1979; Sege & Peterson, 1980; Vincent et al., 1981).

MHC sublocus-analysis indicated that only products from AgB-A contributed significantly to the observed reaction (Table 1). The anti-AgB-B and anti-AgB-C antisera did not react in spite of the fact that they were of high titer (Günther, personal communication). The reactivity of a monoclonal antibody against AgB-A confirmed the importance of the A-sublocus (Fig. 5). However, precipitation with alloantiserum indicated that less than half of the  $\beta_2m$ -AgB-complexes reacted with the monoclonal antibody. This points to the presence of at least one other  $\beta_2m$ -binding alloantigen, in agreement with other reported data supporting more than one type of class 1 antigens in the rat (Blankenhorn et al., 1978; Kunz et al., 1982; Misra et al., 1982; Natori et al., 1979; Sporer et al., 1979). This result further emphasizes the potential practical use of the heterologous interaction between  $\beta_2m$  and  $\beta_2m$ -binding molecules.

The fact that the class 1 antigen heavy chain has approximately the same binding constants for homologous and heterologous  $\beta_2m$  (Church et al., 1982; this report) has several implications. From a technical point of view it means that MHC class 1 antigens can be specifically radiolabelled through  $\beta_2m$ -exchange, also in species where purified MHC antigens are not available. More important, our data suggest that the interacting sites on  $\beta_2m$  and the class 1 antigen heavy chain are evolutionally conserved. This would be expected if the interaction is of functional importance, since a mutation that changes the properties of the interacting site in  $\beta_2m$  would usually require a simultaneous and favourable mutation in the corresponding site of the  $\beta_2m$ -binding MHC antigen heavy chain in order to maintain the binding affinity. Such parallel mutations are probably rare.

As mentioned above,  $\beta_2m$  is closely related to several molecules of considerable immunological significance, which gives evolutionary investigations of the protein an added scientific potential. Thus, we would like to draw attention to the possibility of using the heterologous exchange of  $\beta_2m$  in class 1 antigen heavy chains in such evolutionary studies. Inhibition of this interaction with solubilized tissues from various species has already contributed to the detection of  $\beta_2m$ -like molecules in early vertebrates and invertebrates (Shalev et al., 1981).

#### ACKNOWLEDGEMENT

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# Binding of $\beta_2$ -Microglobulin by Heterologous Sera

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Purified human, rat or guinea-pig  $\beta_2$ -microglobulin ( $\beta_2m$ ) was mixed with sera from guinea-pig, rat, mouse, rabbit, horse, goat, cow, rhesus monkey or man. The mixtures were incubated at 37°C for various lengths of time. When the sera were separated by gel-chromatography on Sephadex G-200,  $\beta_2m$  was traced not only in 'free' form but also in fractions with higher molecular weights. Evidence is presented suggesting that heterologous  $\beta_2m$  binds to  $\beta_2m$ -containing molecules in sera by exchange with the homologous counterpart.

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Interest has focused on the low molecular weight protein  $\beta_2$ -microglobulin ( $\beta_2m$ ) [3], mainly because [for references see 2, 20]: (1)  $\beta_2m$  is structurally related to immunoglobulins and has been called a 'free immunoglobulin domain'; (2)  $\beta_2m$  is a constant part of the serologically defined strong transplantation antigens in man (HLA-A, -B and -C antigens), mouse (H-2 K, D and L antigens), and several other species; (3)  $\beta_2m$  is also claimed to be associated with several other molecules on cell surfaces and in biological fluids; (4) studies with antibodies directed against  $\beta_2m$  have indicated a role for  $\beta_2m$  in the mechanism of lymphocyte activation; (5) the  $\beta_2m$  concentration in biological fluids changes in various pathological conditions, notably diseases of the kidney, inflammatory diseases, and various malignancies.

One group showed that human  $\beta_2m$  binds to lymphocytes from mouse, rat and guinea-pig [1] and that the receptor for human  $\beta_2m$  on mouse lymphocytes consists, at least partly, of H-2 antigen molecules [10]. This report demonstrates a similar phenomenon, namely that  $\beta_2m$  binds to components in heterologous sera.

## MATERIALS AND METHODS

$\beta_2m$  and antisera against  $\beta_2m$ . Human  $\beta_2m$  [3], guinea-pig  $\beta_2m$  [5] and rat  $\beta_2m$  [16] were purified in this

laboratory. We used one rabbit anti-human  $\beta_2m$  [3], one goat anti-human  $\beta_2m$  (prepared as in Ref. 4), one goat anti-guinea-pig  $\beta_2m$  [4], one goat anti-rat  $\beta_2m$  [16] and one rabbit anti-rat  $\beta_2m$  [16].

*Sera.* We used sera from nine mammalian species belonging to five orders: man, rhesus monkey (Primates), guinea-pig, rat, mouse (Rodentia), rabbit (Lagomorpha), horse (Perissodactyla), goat and cow (Artiodactyla).

*Radioimmunoassay (RIA) of  $\beta_2m$ .*  $\beta_2m$ 's were  $^{125}I$ -radio-labelled by the chloramine-T method [9] and separated from free  $^{125}I$  by gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). RIA for  $\beta_2m$  was performed as described by Plesner *et al.* [22], using polyethyleneglycol exclusion to separate immune complexes from unbound  $\beta_2m$ . Homologous  $\beta_2m$  RIAs were based on the reactions between  $^{125}I$ -labelled human, rat or guinea-pig  $\beta_2m$ 's and their specific goat antisera, respectively; heterologous RIA [8] was based on the reaction between rabbit anti-rat  $\beta_2m$  and  $^{125}I$ -labelled human  $\beta_2m$  (heterologous RIA-1) or the reaction between rabbit anti-human  $\beta_2m$  and  $^{125}I$ -labelled guinea-pig  $\beta_2m$  (heterologous RIA-2). The two heterologous RIAs were selected as being highly sensitive for demonstration of cross-reactions. The detection limit of the homologous RIAs varied between batches of labelled protein (0.2–5  $\mu g/l$ ).

*Incubation of  $\beta_2m$  with heterologous serum.* Purified  $\beta_2m$  was mixed with heterologous serum, usually 1–3  $\mu g/ml$  serum, and incubated at 37°C for 1–2 days, unless otherwise stated. The incubations were interrupted by the application of the mixtures to columns of Sephadex G-200.

*Separation of serum by gel chromatography.* Columns of Sephadex G-200 (Pharmacia), with lengths of 90–130 cm and volumes of 60–180 ml, were equilibrated with 0.02 M Tris-HCl buffer + 0.15 M NaCl + 0.02%



$\text{NaN}_3$ , pH 8.0, and used for serum separation. In a few experiments a larger Sephadex G-200 column ( $5 \times 135$  cm) was used, equilibrated with the same buffer. The protein distribution was determined by measuring the absorbance at 280 nm.  $\beta_2\text{m}$  was traced by homologous or heterologous RIA.

## RESULTS

Rat  $\beta_2\text{m}$  mixed with horse serum and incubated at  $37^\circ\text{C}$  for 24 h and then chromatographed on Sephadex G-200, was not solely recovered in 'free' form but also in two high molecular weight peaks, B and C, with average  $K_{\text{av}}$  values of 0.09 and 0.42, respectively (Fig. 1). The 'peak of 'free'  $\beta_2\text{m}$  is designated D.

### *Influence of incubation temperature, incubation time, and $\beta_2\text{m}$ concentration on the complex formation of rat $\beta_2\text{m}$ in horse serum*

**Incubation temperature** (24 h;  $2\mu\text{g}$  rat  $\beta_2\text{m}/\text{ml}$ ). With incubation at  $4^\circ\text{C}$ , or less, all rat  $\beta_2\text{m}$  was eluted in 'free' form. Rat  $\beta_2\text{m}$  could also be detected as a peak B after incubation at  $20^\circ\text{C}$ .

However, after incubation at  $37^\circ\text{C}$ , the amounts of rat  $\beta_2\text{m}$  present in peak B were markedly increased; in addition, rat  $\beta_2\text{m}$  was also detected as a peak C.

**Incubation time** ( $37^\circ\text{C}$ ;  $2\mu\text{g}$  rat  $\beta_2\text{m}/\text{ml}$ ). If horse serum was separated immediately after mixture with rat  $\beta_2\text{m}$ , the rat  $\beta_2\text{m}$  was only recovered as a peak D. Already after 3 h of incubation, significant amounts of complexed rat  $\beta_2\text{m}$  could be detected. Incubation times of more than 2 days increased the amounts of complexed rat  $\beta_2\text{m}$  only to a small extent. However, prolonged incubation time led to decrease of peak B and increase of peak C.

**Rat  $\beta_2\text{m}$  concentration** ( $37^\circ\text{C}$ ; 24 h). At  $0.4\mu\text{g}$  rat  $\beta_2\text{m}/\text{ml}$ , complexed rat  $\beta_2\text{m}$  was barely detectable (in these experiments, the detection limit of the RIA lay between 2 and  $5\mu\text{g}/\text{l}$ ). At  $2\mu\text{g}/\text{ml}$ , about one fourth of the rat  $\beta_2\text{m}$  could be detected in well-defined peaks B and C. At  $10\mu\text{g}/\text{ml}$ , the absolute amounts of complexed rat  $\beta_2\text{m}$  further increased, but its percentage decreased.

On the basis of these experiments, we used incubations at  $37^\circ\text{C}$  for 1–2 days with 1–3  $\mu\text{g}$   $\beta_2\text{m}/\text{ml}$  in most of the following experiments.

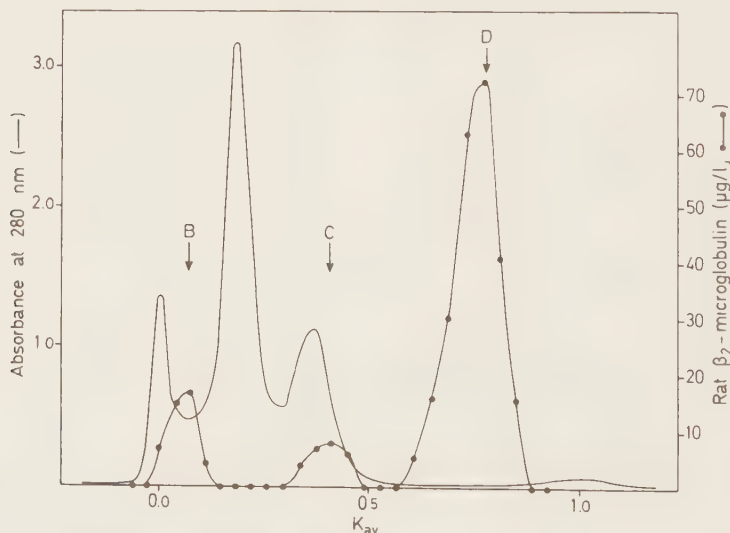


FIG. 1. Horse serum was incubated with rat  $\beta_2\text{m}$  ( $2\mu\text{g}/\text{ml}$ ) at  $37^\circ\text{C}$  for 24 h.  $450\mu\text{l}$  of the mixture was applied to a column ( $1.1 \times 93$  cm) of Sephadex G-200, equilibrated with  $0.02\text{ M}$  Tris-HCl, pH 8.0, containing  $0.15\text{ M}$  NaCl and  $0.02\%$   $\text{NaN}_3$ . The flow rate was  $2\text{ ml}/\text{h}$  and  $1\text{-ml}$  fractions were collected. The protein distribution was measured as absorbance at 280 nm. Rat  $\beta_2\text{m}$  was traced by homologous rat  $\beta_2\text{m}$  RIA. For convenience, the elution volumes were transformed to  $K_{\text{av}}$  values. The  $\beta_2\text{m}$  peaks are designated with capital letters (B, C, D) as defined in the text.

*Interaction of  $\beta_2\text{m}$  with various heterologous mammalian sera*

We screened the interaction of human, rat or guinea-pig  $\beta_2\text{m}$  with several mammalian sera. Peaks of complexed material were found in three regions after Sephadex G-200 gel chromatography: (A) at  $V_0$  (peak  $K_{av} = 0$ ); (B) between the first and second protein peaks (peak  $K_{av} = 0.08-0.15$ ); and (C) around the third protein peak (peak  $K_{av} = 0.32-0.42$ ). In addition, we frequently detected  $\beta_2\text{m}$  peaks between peaks C and D. These were not as readily reproducible as the others. They were thought to represent

self-association of  $\beta_2\text{m}$  and were not further investigated.

The peaks were scored as — (not detected); (+) ( $\beta_2\text{m}$  detected but peak not distinct, because of low amounts, close to the detection limit, or poor resolution from background level); + (distinct, well-defined  $\beta_2\text{m}$ -peak containing < 10% of total  $\beta_2\text{m}$ ); or ++ ( $\beta_2\text{m}$  peak containing > 10% of total  $\beta_2\text{m}$ ). Table I gives a compilation of the results. It also gives the elution profile of homologous  $\beta_2\text{m}$  in mouse, horse and cow sera, as detected by heterologous RIA. Of the various sera, only mouse serum could inhibit the homologous rat  $\beta_2\text{m}$  RIA. The inhibitions

TABLE I. Binding of  $\beta_2\text{m}$  by heterologous sera

| Serum              | $\beta_2\text{m}$ addition   | Peak A | Peak B | Peak C |
|--------------------|------------------------------|--------|--------|--------|
| Man                | Rat $\beta_2\text{m}$        | +      | (+)    | (+)    |
|                    | Guinea-pig $\beta_2\text{m}$ | (+)    | (+)    | ++     |
| Rhesus monkey      | Human $\beta_2\text{m}$      | (+)    | (+)    | (+)    |
|                    | Rat $\beta_2\text{m}$        | (+)    | (+)    | +      |
|                    | Guinea-pig $\beta_2\text{m}$ | (+)    | (+)    | +      |
| Rat                | Human $\beta_2\text{m}$      | (+)    | (+)    | (+)    |
|                    | Guinea-pig $\beta_2\text{m}$ | (+)    | —      | +      |
| Guinea-pig         | Human $\beta_2\text{m}$      | (+)    | —      | +      |
|                    | Rat $\beta_2\text{m}$        | +      | —      | ++     |
| Mouse              | Human $\beta_2\text{m}$      | —      | ++     | ++     |
|                    | Rat $\beta_2\text{m}$        | —      | ++     | ++     |
|                    | Guinea-pig $\beta_2\text{m}$ | —      | ++     | ++     |
| Heterologous RIA-1 |                              | —      | ++     | ++     |
| Rabbit             | Human $\beta_2\text{m}$      | (+)    | +      | (+)    |
|                    | Rat $\beta_2\text{m}$        | (+)    | (+)    | +      |
|                    | Guinea-pig $\beta_2\text{m}$ | +      | (+)    | ++     |
| Cow                | Human $\beta_2\text{m}$      | (+)    | (+)    | (+)    |
|                    | Rat $\beta_2\text{m}$        | —      | (+)    | +      |
|                    | Guinea-pig $\beta_2\text{m}$ | —      | —      | (+)    |
| Heterologous RIA-1 |                              | —      | —      | (+)    |
| Heterologous RIA-2 |                              | —      | —      | —      |
| Horse              | Human $\beta_2\text{m}$      | —      | ++     | —      |
|                    | Rat $\beta_2\text{m}$        | —      | ++     | +      |
|                    | Guinea-pig $\beta_2\text{m}$ | —      | ++     | ++     |
| Heterologous RIA-1 |                              | —      | ++     | ++     |
| Goat               | Human $\beta_2\text{m}$      | (+)    | (+)    | ++     |
|                    | Rat $\beta_2\text{m}$        | +      | (+)    | ++     |
|                    | Guinea-pig $\beta_2\text{m}$ | +      | +      | ++     |

Human, rat or guinea-pig  $\beta_2\text{m}$  was added to the various sera to final concentrations of 1–3  $\mu\text{g}/\text{ml}$ . The mixtures were incubated at 37°C for 1–2 days and then chromatographed on columns of Sephadex G-200. The  $\beta_2\text{m}$  content of the fractions was determined by RIAs homologous to the species of  $\beta_2\text{m}$  added.  $\beta_2\text{m}$ -containing peaks were integrated to measure their total  $\beta_2\text{m}$  content. The presence of high molecular weight  $\beta_2\text{m}$  peaks in regions A, B, and C are indicated by a score: — = not detected; (+) =  $\beta_2\text{m}$  detected but peak not distinct; + = distinct  $\beta_2\text{m}$  peak containing < 10% of total  $\beta_2\text{m}$ ; ++ =  $\beta_2\text{m}$ -peak containing > 10% of total  $\beta_2\text{m}$ . In a few experiments, sera were separated on Sephadex G-200 after no addition of heterologous  $\beta_2\text{m}$ . The  $\beta_2\text{m}$  distribution was then determined by heterologous RIA.

caused by such cross-reactive materials, however, were low compared with that caused by complexed rat  $\beta_2\text{m}$ . Incubations with mouse or horse sera gave similar elution profiles, with prominent peak B, more (mouse) or less (horse) prominent peak C, and no peak A (cf. Fig. 1). In the other sera, complexed  $\beta_2\text{m}$  was preferentially detected in region C with various degrees of minor amounts in the other two regions (cf. Fig. 2). The elution profiles for the various  $\beta_2\text{m}$ 's were very similar within each serum. It should be noted that the location of both peak B and peak C varied between, but was reproducible within, species.

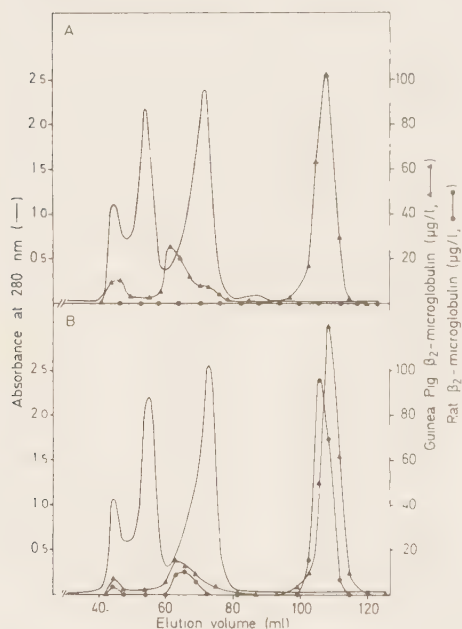


FIG. 2. Guinea-pig serum (A). Guinea-pig serum incubated at 37°C for 24 h with rat  $\beta_2\text{m}$  (1  $\mu\text{g}/\text{ml}$ ) (B). 750  $\mu\text{l}$  of each was separated on a column (1.4  $\times$  90 cm) of Sephadex G-200 superfine, equilibrated with 0.02 M Tris-HCl + 0.15 M NaCl + 0.02% NaN<sub>3</sub>, pH 8.0. The flow rate was 2 ml/h, and 1.5-ml fractions were collected. The protein distribution was measured as absorbance at 280 nm. The rat and guinea-pig  $\beta_2\text{m}$  in the eluates were traced by the respective homologous  $\beta_2\text{m}$  RIAs.

#### *Re-chromatography of selected peaks*

Fractions comprising peak B or C of rat  $\beta_2\text{m}$ -containing horse serum were pooled, concentrated, and re-chromatographed on Sephadex

G-200. All of the peak C material eluted again in region C. More interesting, only a part of the peak B material emerged in region B, the rest eluting at region C.

When peak C of rat  $\beta_2\text{m}$ -incubated rabbit serum was similarly re-chromatographed in large amounts, the bulk of the material emerged in region C. However, a small peak A also appeared.

#### *Effect of guinea-pig $\beta_2\text{m}$ on the complex formation of rat $\beta_2\text{m}$ in guinea-pig serum*

Guinea-pig serum (Fig. 2A) and guinea-pig serum incubated with rat  $\beta_2\text{m}$  (Fig. 2B) were separated by Sephadex G-200 gel chromatography. The rat and guinea-pig  $\beta_2\text{m}$  content of the fractions were determined by the respective homologous RIAs. It is notable that the elution of complexed rat  $\beta_2\text{m}$  coincides with the elution of high molecular weight guinea-pig  $\beta_2\text{m}$  complexes (Fig. 2B). When we added large amounts of purified guinea-pig  $\beta_2\text{m}$  (1 mg/ml) to the incubation mixture of rat  $\beta_2\text{m}$  and guinea-pig serum, the rat  $\beta_2\text{m}$  of the resulting G-200-separation was almost exclusively confined to peak D (not shown).

## DISCUSSION

The results presented in this paper indicate that purified  $\beta_2\text{m}$  binds to components in heterologous sera. Bound  $\beta_2\text{m}$  is detected as peaks in three regions of the various sera (regions A–C as defined above).

The binding profiles show striking similarities to the distribution profile of homologous  $\beta_2\text{m}$  in the various sera, as these have been reported for mouse [13, 18], man [17], rat [16], rabbit [7, Björck, L. & Berggård, I., unpublished] and guinea-pig [present paper]. Moreover, in horse serum, evaluated by heterologous RIA, the  $\beta_2\text{m}$  distributed in the same pattern as the heterologous  $\beta_2\text{m}$ -binding profile (Table I). When  $^{125}\text{I}$ -labelled rat  $\beta_2\text{m}$  was mixed with rat serum incubated at 37°C for 24 h and then separated on Sephadex G-200, we earlier found that the radioactivity would be recovered in three peaks, corresponding to the rat  $\beta_2\text{m}$  detectable in rat serum with RIA [16]. These results were interpreted as due to an exchange of the rat  $\beta_2\text{m}$  in the high molecular weight  $\beta_2\text{m}$ -containing materials with  $^{125}\text{I}$ -labelled rat

$\beta_2m$  [16]. Thus, it seems probable that heterologous  $\beta_2m$  binds to high molecular weight  $\beta_2m$ -containing molecules through exchange with the homologous counterpart.

Further support for the exchange hypothesis was obtained in experiments in which rat  $\beta_2m$  was bound by guinea-pig serum (Fig. 2). Addition of large amounts of purified guinea-pig  $\beta_2m$  almost completely blocked the binding of rat  $\beta_2m$  to guinea-pig serum. In addition, Fig. 2B shows that similar amounts of rat and guinea-pig  $\beta_2m$  leads to quantitatively comparable distribution profiles of the two proteins in guinea-pig serum. This suggests that the  $\beta_2m$ -binding materials in peaks A and C have affinities of the same magnitude for rat and guinea-pig  $\beta_2m$ , respectively. Thus, our data indicate that there is an evolutionary conservatism in the binding sites of  $\beta_2m$  and the complementary sites of the  $\beta_2m$ -binding molecules, and that the heterologous binding represents a form of cross-reactivity.

Discussing the mechanisms for the binding of human  $\beta_2m$  to histocompatibility antigens on mouse and human lymphocytes, Hester *et al.* [10] suggested two possibilities: (1) more than one  $\beta_2m$  molecule might bind to a single histocompatibility antigen heavy chain, and (2) there exist on the cell surface histocompatibility antigens lacking  $\beta_2m$  and therefore capable of binding  $\beta_2m$ . The two models do not seem to be probable candidates to explain the binding of  $\beta_2m$  by heterologous sera. Serum contains considerable amounts of unbound  $\beta_2m$ , and such mechanisms would accordingly be expected to be saturated. Thus, an exchange model based on dynamic equilibrium reactions appears to be a more probable explanation of this phenomenon. Exchange could also be the mechanism behind the cytophilic properties of human  $\beta_2m$  for heterologous or activated homologous lymphocytes.

The rat  $\beta_2m$ -containing peak B of horse serum was unstable and gave a peak in region C when re-chromatographed. This lack of stability, also supported by the temporal binding pattern, has previously been noted for the homologous  $\beta_2m$  peak B of mouse serum [13]. In that report, the resulting peak C material had a structure closely resembling H-2 antigens but lacked alloantigenic activity. A small part of the rat  $\beta_2m$ -containing peak C of rabbit serum eluted as peak A material when re-chromato-

graphed. One may speculate that a flow—peak B  $\rightarrow$  peak C  $\rightarrow$  peak A—exists, and a heterologous binding of  $\beta_2m$  to peak B could then explain our results. With homologous RIA, significant amounts of peak B  $\beta_2m$  have been found in the mouse [13, 18] and man [17], but not in the rabbit [7; Björck, L. & Berggård, I., unpublished], or guinea-pig [present paper]. We could not detect peak B  $\beta_2m$  in rat serum [16]; recently, another group found a peak B  $\beta_2m$  in sera of Hooded Lister rats [12]. The reason for this is unknown, but possible explanations are that peaks B of the latter species are rapidly degraded to peak C materials, or that antigenic  $\beta_2m$  determinants of these materials are hidden.

A second type of molecules that could be responsible for heterologous  $\beta_2m$ -binding are the major histocompatibility antigens (HLA-A, -B, and -C antigens and their animal homologues) [10]. This is also suggested by studies on man/mouse cell hybrids [6, 11]. Mouse  $\beta_2m$  apparently could replace human  $\beta_2m$  in the HLA antigens expressed on the cell surface of the hybrid cells. Our own recent demonstration that purified papain-solubilized AgB antigens can bind heterologous  $\beta_2m$  further supports this notion [15]. These materials would be expected to elute in region C. Their different tendency to aggregate and form dimers [26] could explain the different location of the peak C, responsible for heterologous  $\beta_2m$ -binding, of the various species. Peak C of guinea-pig serum would then mainly consist of the dimer, with only small amounts of monomer (Fig. 2A), whereas peak C of horse serum represents the monomer (Fig. 1). However, so far we have not been able to precipitate, with relevant alloantisera,  $^{125}I$ -labelled guinea-pig or human  $\beta_2m$  bound to peak C of serum from inbred Wistar-Furth rats (unpublished data; cf. Ref. 15). This very preliminary observation suggests that the main part of the  $\beta_2m$ -binding molecules in heterologous sera are devoid of alloantigenic activity. Such molecules could possibly also explain part of the cytophilic properties of  $\beta_2m$  [23].

$\beta_2m$ , immunoglobulins, and major histocompatibility antigens appear to share a common evolutionary origin [19, 21, 24, 25]. Thus, it is of interest to trace, isolate, and characterize  $\beta_2m$  in lower species. The heterologous RIA described by Gordon & Kindt [8] should be a valuable tool in such studies. The heterologous binding of  $\beta_2m$ , as described in the present



paper, could be a helpful supplement to the heterologous RIA. This is exemplified by cow serum (Table I), where two sensitive heterologous RIAs failed to demonstrate unambiguously anything but 'free'  $\beta_2$ m. A clear peak C material, however, was demonstrated by the heterologous binding technique.

$\beta_2$ m is apparently important for the conformation of major histocompatibility antigens [14, 26]; the loss of  $\beta_2$ m is often followed by diminished alloantigenic activity. It would be interesting to investigate whether heterologous  $\beta_2$ m exchange in  $\beta_2$ m-containing molecules leads to altered conformation, detectable with biochemical or immunological methods.

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BINDING OF MAMMALIAN  $\beta_2$ -MICROGLOBULIN BY GLYCOPROTEINS IN FISH SERUM

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## ABSTRACT

The results demonstrate the presence in cod serum of  $\beta_2$ -microglobulin ( $\beta_2m$ )-binding molecules. Upon fractionation on Sephadex G-200, the bound  $\beta_2m$  appears mainly in the void volume, but also as a minor peak with the apparent size of albumin. The complexes show affinity to Con A-Sepharose and Lens culinaris lectin-Sepharose, respectively, indicating that they contain glycoproteins. Because of the high molecular weight of the  $\beta_2m$ -containing complexes they can be separated from unbound  $\beta_2m$  by poly-ethylenglycol (PEG-6000) precipitation, thus allowing rapid analysis. The binding is temperature-dependent. At 37°C, maximum binding is reached after about 2 hours. The dissociation is considerably slower, complete dissociation taking about 2 days. According to Scatchard analysis, the association constant is of the order  $2 \times 10^9 \text{ M}^{-1}$ . The binding is sensitive to denaturing agents and high salt concentrations. Optimum binding is seen at neutral pH and the  $\beta_2m$ -binding activity is heat-labile at 50°C. The binding of heterologous  $\beta_2m$  by cod serum can be used as a cross-reactive assay specific for the detection of  $\beta_2m$ . Whereas unlabelled human, guinea pig, and rat  $\beta_2m$ , respectively, give similar inhibition, higher concentrations of rabbit  $\beta_2m$  are needed for the same degree of inhibition. Radiolabelled mammalian  $\beta_2m$  also binds to cod splenocytes. Thus, the  $\beta_2m$ -binding molecules in cod serum may represent products shedded from the cell surface.

## INTRODUCTION

The immunoglobulin-like domain seems to be a common feature of many important molecules of the immune system. Thus, several molecules contain regions with amino acid sequence-homology to the immunoglobulin domains, indicating a common evolutionary origin. Apart from the immunoglobulins themselves,  $\beta_2$ -microglobulin ( $\beta_2m$ ) (Peterson et al., 1972; Smithies & Poulik, 1972), major histocompatibility (MHC) Class I (Orr et al., 1979; Trägårdh et al., 1979) and Class II (Larhammar et al., 1981; cf. Robertsson, 1982) -antigens, Thy-1-antigen (Cohen et al., 1981), and Thymopoietin (Hahn & Hamburger, 1981) have been reported to belong to this protein family.  $\beta_2m$  is a constant part not only of MHC Class I-antigens (HLA-A, B, C-antigens in man; H-2 K, D, L-antigens in mouse; AgB-antigens in rat; etc.), but also of TL (Östberg et al., 1975; Vitetta et al., 1975), Qa-1 (Stanton & Hood, 1980), and Qa-2 (Michaelson et al., 1977) antigens. In addition,  $\beta_2m$ -determinants have been reported to be associated with antigen-specific (Lamb et al., 1981) and -non-specific (Armerding et al., 1975) lymphocyte interaction factors, with various serum molecules (Callahan et al., 1976; Katz, 1978; Kvist & Peterson, 1978; Natori et al., 1976), and with the male-specific H-Y-antigen (Fellous et al., 1978). Further structural studies on these latter molecules will probably add more members to the above-mentioned protein family (Soloski et al., 1981). These data, taken together, form a strong case for the study of  $\beta_2m$  and  $\beta_2m$ -binding molecules in non-mammalian species.

MHC Class I-antigen- and  $\beta_2m$ -homologues in birds are to date the only non-mammalian homologues of these molecules to have been unambiguously demonstrated (Kvist et al., 1978; Vitetta et al., 1977). However, func-

tional tests indicate the presence of analogous molecules even in invertebrates (Cooper, 1981). Also, a recent report demonstrates " $\beta_2m$ -activity" in several non-mammalian vertebrates and invertebrates (Shalev et al., 1981). The interaction between  $\beta_2m$  and  $\beta_2m$ -binding molecules has apparently been well conserved during evolution. Thus, many mammalian  $\beta_2m$ -binding molecules, like MHC Class I-antigens (Church et al., 1982; Schmidt et al., 1981; Lögdberg et al., 1980), serum molecules (Lögdberg et al., 1981b; Vincent et al., 1981), and cell surface " $\beta_2m$ -receptors" (Anderson et al., 1975; Lögdberg et al., 1981a; Sege et al., 1979; Sege & Peterson, 1980) can bind both homologous and heterologous  $\beta_2m$ . It is therefore possible that non-mammalian homologues of  $\beta_2m$ -binding molecules may be able to bind mammalian  $\beta_2m$ . Thus, we have screened sera from non-mammalian vertebrates for  $\beta_2m$ -binding capacity. In all tested species, including birds, reptiles, bonyfishes, elasmobranches and cyclostomes, we could detect glycoproteins binding radiolabelled human  $\beta_2m$ . Serum from codfish contained particularly prominent  $\beta_2m$ -binding activity, and was selected for the further studies presented in this paper.

## MATERIALS AND METHODS

### $\beta_2$ -microglobulins.

Human (Berggård & Bearn, 1968), rat (Lögdberg et al., 1979), guinea pig (Cigén et al., 1978), and rabbit (Björck & Berggård, 1981)  $\beta_2m$ , respectively, were purified in this laboratory.

### Codfish: serum and splenocytes.

Codfishes were provided by local fishermen. To obtain serum, they were bled through aortic incision. The spleens were teased and minced in phosphate-buffered saline, and then filtered through double gauze, in order to obtain single cell suspensions.

### Radioimmunoassay (RIA).

$\beta_2m$  was  $^{125}I$ -radiolabelled by the chloramin-T-method (Greenwood et al., 1963). The RIA-method for human  $\beta_2m$ , described by Plesner et al. (1975), was adapted to rat  $\beta_2m$  (Lögdberg et al., 1979). RIA-buffer: 0.1 M phosphate-buffer, pH 7.4, containing 0.02%  $NaN_3$  and 0.1% bovine serum albumin. Polyethylenglycol-exclusion (PEG 6000, Kebo AB, Stockholm) was used to separate immune complexes from unbound  $\beta_2m$ .

### Binding of $\beta_2$ -microglobulin to serum.

Radiolabelled  $\beta_2m$  was mixed with cod serum in a total volume of 0.5 ml RIA-buffer. The mixtures were incubated at 37°C for the times indicated in the text. The degree of binding was then analysed, either after separation of the mixtures by gel chromatography, or after addition of 10% PEG 6000 followed by centrifugation. In the presence of PEG 6000 and 12.5% normal ox serum (as carrier), bound  $\beta_2m$  is precipitated. Binding experiments using this kind of detection, were always performed in duplicates (duplicate variation ~4%).

### Binding of $\beta_2$ -microglobulin to cod splenocytes.

Radiolabelled  $\beta_2m$  and cod splenocytes ( $1 - 10 \times 10^6$ ) were mixed in a total volume of 0.1 - 1 ml of RIA-buffer. After 2 hours of incubation at 37°C, the cells were spun down, washed at least twice, and then subjected to  $\gamma$ -counting. Excess amounts of unlabelled  $\beta_2m$  were added to some incubations, in order to measure non-specific binding.

### Determination of binding constant.

One  $\mu$ l of cod serum was incubated with increasing concentrations of radio-labelled human  $\beta_2m$ . After 2 hours, bound  $\beta_2m$  was precipitated by addition of PEG 6000. From the radioactive counts, the concentrations of free and bound  $\beta_2m$  could be determined. Such data were used for Scatchard analyses (Scatchard, 1949).

### Gel chromatography.

Incubation mixtures (cod serum +  $\beta_2m$ ) were applied to columns of Sephadex G-200-superfine (Pharmacia, Uppsala), with lengths of 90-130 cm and volumes of 60-140 ml, equilibrated with 0.02 M Tris-HCl, pH 8.0, + 0.15 M NaCl + 0.02%  $NaN_3$ . Flow rate was 1.2 - 1.6 ml/h. The eluent was analysed for protein by reading the absorbance at 280 nm and for  $\beta_2m$  by  $\gamma$ -counting ( $^{125}I$ - $\beta_2m$ ) or  $\beta_2m$ -RIA, respectively.

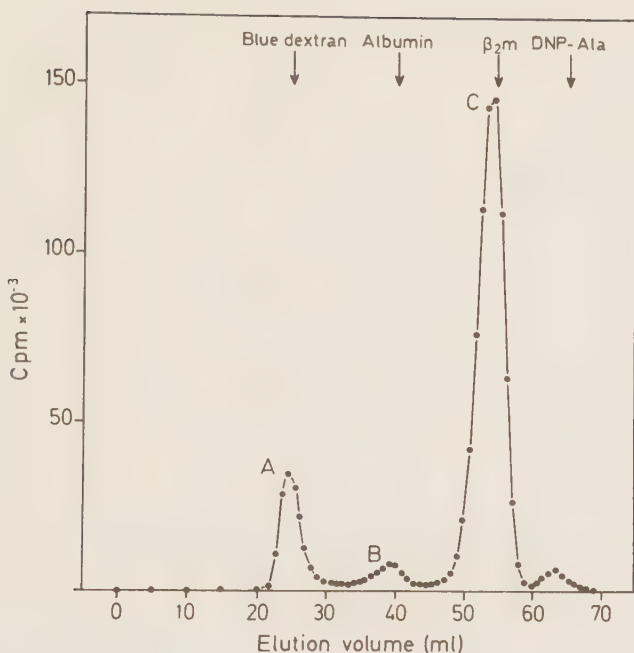
### Affinity chromatography.

Samples to be analysed were adjusted to 0.05 M Tris-HCl, pH 7.7 + 1 M NaCl + 1 mM  $MgCl_2$ ,  $CaCl_2$  and  $MnCl_2$  (Starting buffer), and then applied to columns of Con A-Sepharose or Lens culinaris lectin-Sepharose, respectively (both Pharmacia), prepared in pasteur pipettes ( $\sim 1$ ml), and equilibrated with Starting buffer. The columns were washed with Starting buffer and then eluted with 0.1 M  $\alpha$ -methyl-D-glucoside or  $\alpha$ -methyl-D-mannoside, respectively, in the same buffer.

## RESULTS

### Binding of radiolabelled human $\beta_2$ -microglobulin by high molecular weight glycoproteins in cod serum.

After incubation with cod serum at 37°C, the gel chromatography profile of  $^{125}I$ -labelled human  $\beta_2m$  on Sephadex G-200-superfine was altered (Fig. 1). In addition to a peak corresponding to monomeric  $\beta_2m$ , radioactivity could now also be detected in high molecular weight forms. A major peak was found at the void volume (peak A), sometimes even exceeding the height of the unbound  $\beta_2m$  (peak C). Also, a minor, but well-defined peak (peak B) of  $\beta_2m$ -binding material was detected at an elution volume corresponding to that of albumin. Peaks A and B, but not peak C, bound to Con A-Sepharose affinity columns (Fig. 2). Similarly, cod serum that had been passed through Con A-Sepharose or Lens culinaris lectin-Sepharose columns lost its capacity to bind  $^{125}I$ - $\beta_2m$  (not shown). Instead, the  $\beta_2m$ -binding capacity was recovered when the affinity columns were eluted with the specific sugars.



**Figure 1**

$^{125}\text{I}$ -human  $\beta_2m$  ( $10^6$  cpm;  $\sim 0.1 \mu\text{g}$ ) + cod serum ( $10 \mu\text{l}$ ) in RIA-buffer, total volume  $0.5 \text{ ml}$ , were incubated at  $37^\circ\text{C}$  for 24 hours. The mixture was subsequently subjected to gel chromatography on a Sephadex G-200-superfine column ( $1 \times 90 \text{ cm}$ ). The distribution of radioactivity in the fractions was determined (●—●). Arrows indicate the elution volumes of some column calibration markers. Material from peaks A-C, respectively, was further analysed by affinity chromatography on Con-A-Sepharose (see Fig. 2).



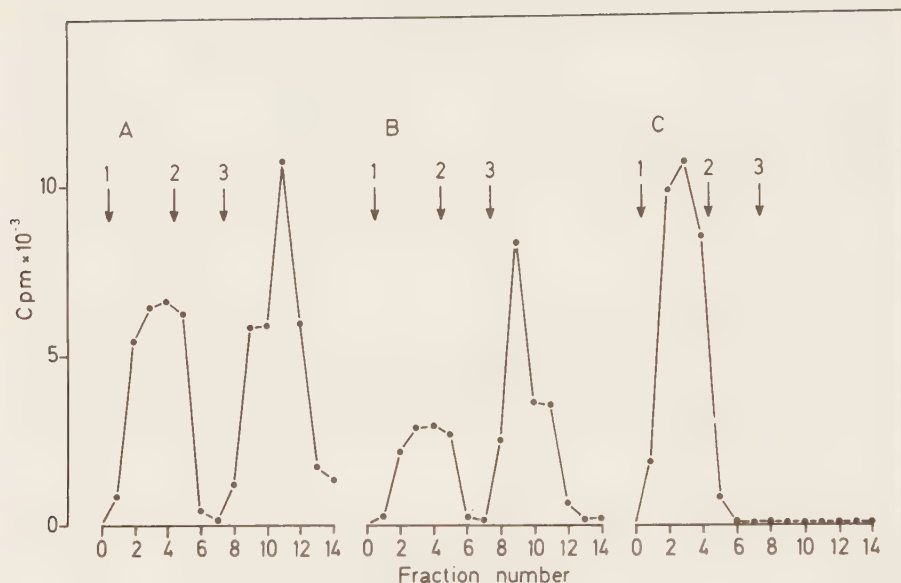


Figure 2

Radioactive material from peaks A-C, as shown in Fig. 1, were applied to small columns of Con A-Sepharose, prepared in pasteur pipettes (~1 ml), after adjustment of the sample buffer to 0.05 M Tris-HCl, pH 7.7, containing 1 M NaCl and 1 mM MgCl<sub>2</sub>, CaCl<sub>2</sub>, and MnCl<sub>2</sub>. After washing, bound radioactivity was eluted with 0.1 M  $\alpha$ -methyl-D-glucoside in the same buffer.

Binding of unlabelled rat  $\beta_2$ m to cod serum was detected, after Sephadex gel chromatography, by rat  $\beta_2$ m-RIA (data not shown). Again  $\beta_2$ m-complexes appeared at the void volume.

Because of the very high molecular weight nature of the complex between  $\beta_2$ m and the  $\beta_2$ m-binding cod molecules, it should be possible to precipitate them by polyethylenglycol-exclusion. Fig. 3 shows that this indeed, is the case. The figure also shows that  $\beta_2$ m-binding can be detected already at very low concentrations of cod serum. Thus, the PEG-precipitation-based binding assay was used in the subsequent experiments.



Figure 3

Titration of the  $\beta_{2m}$ -binding capacity of cod serum. All dilutions were made in RIA-buffer. Cod serum (100  $\mu$ l), in various dilutions, was mixed with  $^{125}$ I-human  $\beta_{2m}$  (100  $\mu$ l) and RIA-buffer (300  $\mu$ l). For background determination (■), 100  $\mu$ l RIA-buffer replaced the cod serum. After incubation at 37°C for 12 hours, bound  $\beta_{2m}$  was precipitated by addition of 10% polyethylenglycol (PEG 6000). Total cpm: 57,000.

#### Binding characteristics: Association, dissociation, and binding constant.

Fig. 4 A demonstrates the time course of binding of excess  $^{125}$ I-human  $\beta_{2m}$  by cod serum at various temperatures. At 37°C, the association is rapid and reaches a plateau after approximately 2 hours. Much slower association occurs at 20°C or 4°C, respectively. The same experiments, but with cod serum in excess of  $^{125}$ I-human  $\beta_{2m}$ , gave similar results.

Dissociation, by addition of excess amounts of unlabelled human  $\beta_{2m}$ , was performed after an initial association time of 2 hours (Fig. 4 B). The dissociation was considerably slower than the association and required more than two days for completion.

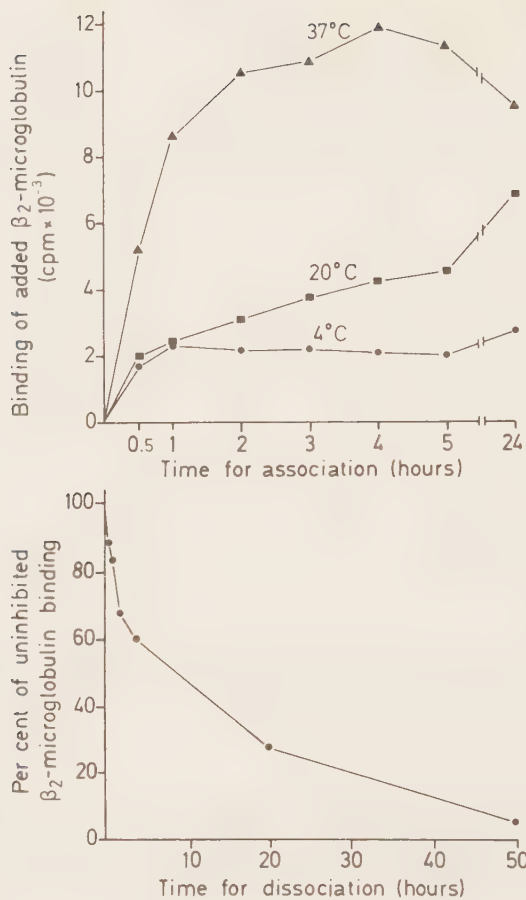


Figure 4

A. Association between cod serum and  $^{125}\text{I}$ -human  $\beta_2\text{m}$ , at various temperatures.  $^{125}\text{I}$ -human  $\beta_2\text{m}$  (44,000 cpm) and 0.2  $\mu\text{l}$  cod serum were incubated in a total volume of 0.5 ml RIA-buffer, for varying lengths of time. Background cpm (control incubation without cod serum) were subtracted and results are presented as  $\Delta$  cpm.

B.  $^{125}\text{I}$ -human  $\beta_2\text{m}$  (42,000 cpm) and 0.2  $\mu\text{l}$  cod serum were incubated in 0.5 ml RIA-buffer at 37°C for 2 hours. Then, excess amounts of unlabelled human  $\beta_2\text{m}$  (10  $\mu\text{g}$ ) were added to some of the incubations. After additional incubation for the indicated time intervals, the incubations were interrupted. Specific binding ( $\Delta$ cpm) was calculated for the dissociated incubations and for control incubations without unlabelled human  $\beta_2\text{m}$ . Results are given as the quota between these two  $\Delta$ cpm-values.

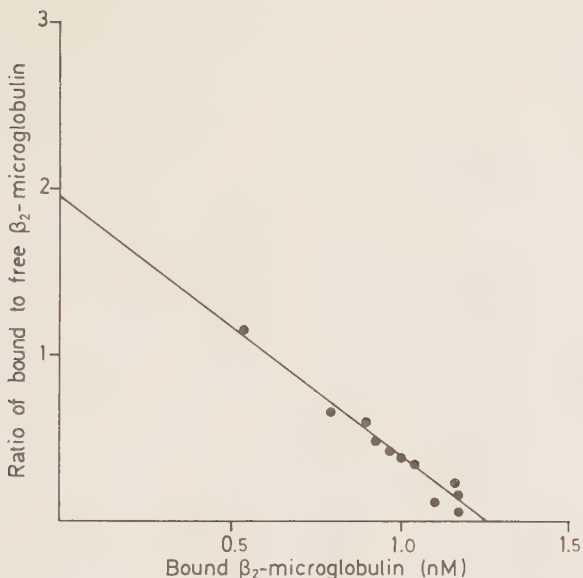


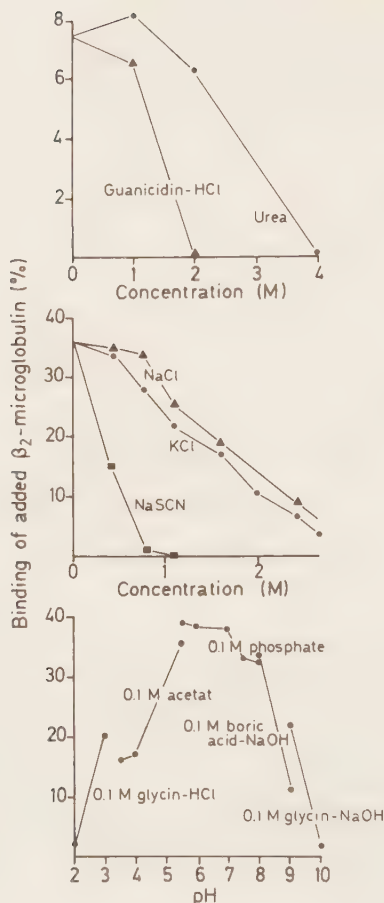
Figure 5

One  $\mu$ l of cod serum was incubated for 2 hours at 37°C with various concentrations of  $^{125}\text{I}$ -human  $\beta_2\text{m}$ , in a total volume of 0.5 ml RIA-buffer. After incubation, the amounts of free and bound  $\beta_2\text{m}$ , respectively, were determined (after correction for the inaccessible fraction of the  $^{125}\text{I}$ - $\beta_2\text{m}$ ). Data were plotted according to Scatchard (1949). Thus, the equilibrium equation can be written:  $B/F = K(A_0 - B)$ , where  $B/F$  is the ratio of bound to free  $\beta_2\text{m}$ ,  $K$  is the binding constant,  $A_0$  is the concentration of binding sites for  $\beta_2\text{m}$ , and  $B$  is the concentration of bound  $\beta_2\text{m}$ . From the intercepts with the axes,  $K$  can be calculated. In the particular example, 1.25 nM of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  was bound at saturation (intercept with abscissa). This value corresponds to  $4.1 \times 10^{17}$  binding sites per liter of cod serum and was used to calculate a binding constant of  $1.6 \times 10^9 \text{ M}^{-1}$ .

A constant amount of cod serum was incubated with various concentration of  $^{125}\text{I}$ -human  $\beta_2\text{m}$ . At high  $\beta_2\text{m}$ -concentrations, the  $\beta_2\text{m}$ -binding capacity was saturated. We used these data for Scatchard analysis, as exemplified in Fig. 5. Multiple determinations on two different pools of cod serum gave binding constants of  $1.6$  and  $3.0 \times 10^9 \text{ M}^{-1}$  (mean =  $2.3 \times 10^9 \text{ M}^{-1}$ ), respectively. The respective number of binding sites per liter of cod serum were  $4.2$  and  $6.0 \times 10^{17}$  (mean =  $5.1 \times 10^{17}$ ).

Effects of denaturing agents, salt concentration and pH, respectively, on the binding of  $^{125}\text{I}$ -human  $\beta_2$ -microglobulin by cod serum.

Fig. 6 A shows the effect of denaturing agents on the binding of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  by cod serum. Guanidine-HCl at 2M or urea at 4M completely abrogates the binding. Similarly, the binding is compromised by high salt concentrations (Fig. 6 B), with complete inhibition at 3M NaCl or KCl. The chaotropic salt NaSCN, was deleterious for the binding, already at 0.8M. Optimum binding was seen at pH 5.5 - 8.0, whereas more extreme pH-values led to decreased binding (Fig. 6 C).



**Figure 6**

Cod serum (0.5  $\mu$ l) and  $^{125}$ I-human  $\beta_2$ m were incubated in a total volume of 0.5 ml at 37°C for 2 hours. A. Incubations in RIA-buffer with various concentrations of Guanidin-HCl or Urea. Total cpm: 87,000. B. Incubations in RIA-buffer containing various concentrations of NaCl, KCl, or NaSCN, respectively. Total cpm: 48,000. C. Incubations at various pH, using different buffer systems. All buffers contained 0.1% bovine serum albumin. Total cpm: 62,000. Results are specific binding ( $\Delta$  cpm) expressed in percentage of total cpm.

The  $\beta_2$ -microglobulin-binding molecules of cod serum are labile at 50°C.

Fig. 7 shows the effects of heating of cod serum, at 50°C, on the subsequent binding of  $^{125}$ I-human  $\beta_2$ m. Apparently, the  $\beta_2$ m-binding molecules in cod serum are quickly inactivated at this temperature, with a half-life of less than 10 minutes.

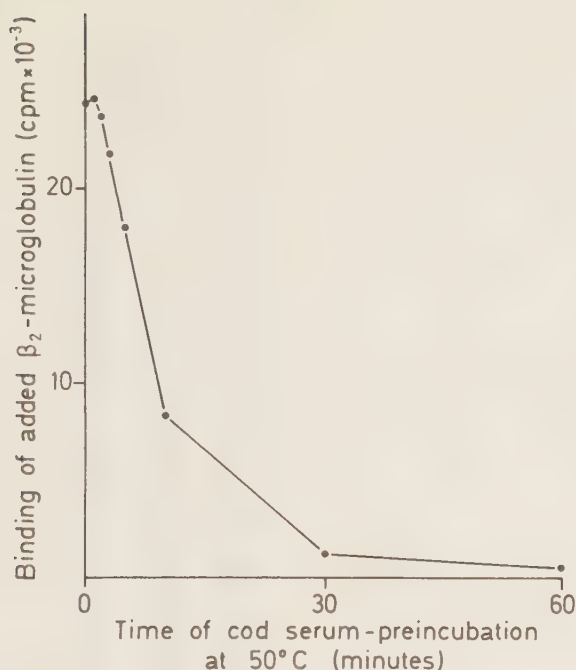


Figure 7

Cod serum was pretreated at 50°C on water-bath for different lengths of time. Then, the binding capacity for  $^{125}\text{I}$ -human  $\beta_2\text{m}$  (42,000 cpm) of this cod serum (1  $\mu\text{l}$ ) was tested in 0.5 ml RIA-buffer. Results are expressed as specific binding ( $\Delta$  cpm).

#### Use of cod serum in a $\beta_2$ -microglobulin-specific, cross-reactive binding assay.

The binding of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  by cod serum was completely abrogated if excess amounts of unlabelled human  $\beta_2\text{m}$  were present during the incubation (Fig. 8). The same amounts of other unlabelled proteins (human immunoglobulin L-chains, human lysozyme, or goat IgG) did not affect the binding. On the other hand, equal concentrations of unlabelled  $\beta_2\text{m}$ -homologues from other species (rat, guinea pig, or rabbit) gave complete inhibition. We investigated the inhibitory capacity of the various  $\beta_2\text{m}$ -homologues at different concentrations (Fig. 9). The inhibition curves of human, rat, or guinea pig  $\beta_2\text{m}$ 's, respectively, were quite similar, whereas higher concentrations of rabbit  $\beta_2\text{m}$  were required to give the same degree of inhibition.



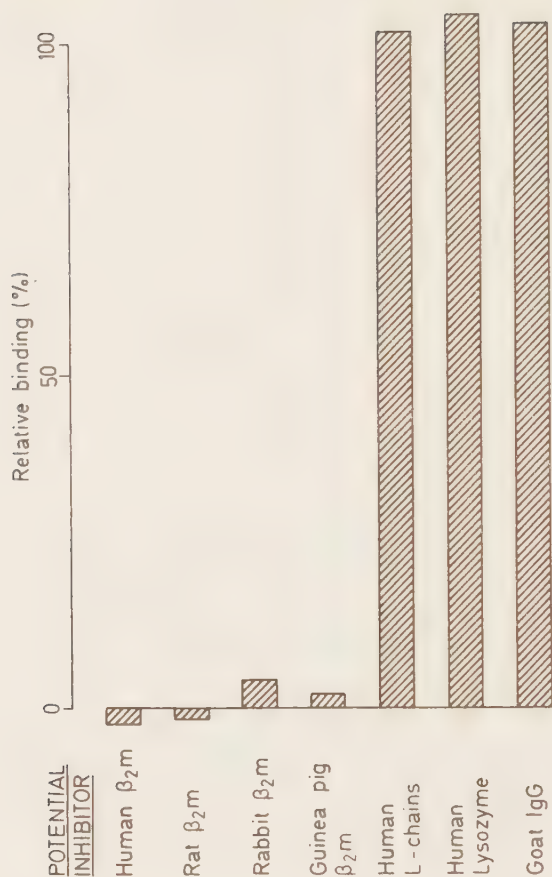


Figure 8

$^{125}I$ -human  $\beta_2m$  and 0.2  $\mu l$  cod serum were incubated for 3 hours, at 37°C, in 0.5 ml RIA-buffer. To some incubations, 5  $\mu g$  of various unlabelled proteins were added. Specific binding ( $\Delta cpm$ ), in the presence of each inhibitor, is presented as the percentage of the specific binding without inhibitor. 100% and 0% binding correspond to 14349 and 2858 cpm, respectively.

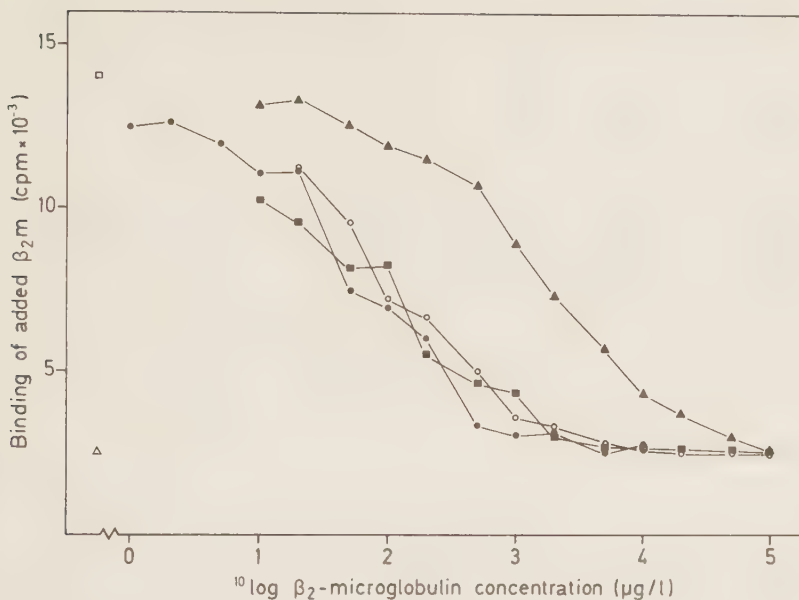


Figure 9

$^{125}\text{I}$ -human  $\beta_2\text{m}$  (90,000 cpm) and 0.2  $\mu\text{l}$  cod serum were incubated in 0.5 ml RIA-buffer, at  $37^\circ\text{C}$ , for 3 hours. Most incubations contained 100  $\mu\text{l}$  of various  $\beta_2\text{m}$ -homologues, human  $\beta_2\text{m}$  (●—●), rat  $\beta_2\text{m}$  (■—■), guinea pig  $\beta_2\text{m}$  (○—○), or rabbit  $\beta_2\text{m}$  (▲—▲), at different concentrations. Results are expressed as PEG-precipitated cpm.

#### Binding of $^{125}\text{I}$ -human $\beta_2\text{-microglobulin}$ by cod splenocytes.

Table 1 demonstrates a moderate binding of  $^{125}\text{I}$ -human  $\beta_2\text{m}$  by cod splenocytes. The binding was specific as it could be inhibited by excess amounts of unlabelled human  $\beta_2\text{m}$ .

Table 1. Binding of  $^{125}\text{I}$ -human  $\beta_2$ -microglobulin by cod splenocytes<sup>a</sup>

| Incubation                                                                                                   | Cell bound $\beta_2\text{m}$ (cpm) <sup>b</sup> |
|--------------------------------------------------------------------------------------------------------------|-------------------------------------------------|
| Splenocytes <sup>c</sup> + $^{125}\text{I}$ -human $\beta_2\text{m}$ <sup>d</sup>                            | 5441 $\pm$ 215                                  |
| Splenocytes + $^{125}\text{I}$ -human $\beta_2\text{m}$<br>+ unlabelled human $\beta_2\text{m}$ <sup>e</sup> | 1182 $\pm$ 45                                   |

<sup>a</sup> Cells were incubated with  $\beta_2\text{m}$  in RIA-buffer at a total volume of 1 ml at 37°C. After 2 hours, cells were spun down, washed three times and then subjected to  $\gamma$ -counting.

<sup>b</sup> Figures are means of double values.

<sup>c</sup>  $4 \times 10^6$  splenocytes per tube.

<sup>d</sup> 162,000 cpm ( $\sim 15$  ng) per tube.

<sup>e</sup> 10  $\mu\text{g}$  per tube.

## DISCUSSION

Much of the scientific interest in  $\beta_2\text{m}$  is based on its association and structural relations with other molecules of biological and immunological significance. The function of  $\beta_2\text{m}$  is far from clear, but investigations focused on the possible interactions between mammalian  $\beta_2\text{m}$  and non-mammalian molecules may add some new insights to the evolution and biological role of  $\beta_2\text{m}$  and the  $\beta_2\text{m}$ -interacting molecules.

This report clearly demonstrates the presence in cod serum of glycoproteins (lectin-affinity) that can bind mammalian  $\beta_2\text{m}$ . Their high molecular weight made it possible to precipitate them by PEG, which formed the basis for a rapid binding-assay. The binding capacity of increasing concentrations of cod serum gave titration curves similar to those of weak anti- $\beta_2\text{m}$  antisera. However, we saw a significant drop in binding capacity, when the cod serum concentration was increased beyond the concentration giving maximum binding. The reason for this is obscure.

Cod serum was only able to bind a minor fraction of the radiolabelled  $\beta_2\text{m}$ . Maximum binding varied between batches of radiolabelled  $\beta_2\text{m}$ . Range: 10 - 50%. Within a given batch of radiolabelled  $\beta_2\text{m}$  the fraction of accessible  $\beta_2\text{m}$  tends to decrease with time. Thus, we believe that the radiolabelling induces subtle changes in the conformation of  $\beta_2\text{m}$ , which render a large part of the radiolabelled  $\beta_2\text{m}$  inaccessible to the  $\beta_2\text{m}$ -binding molecules of cod serum. The ageing of radiolabelled  $\beta_2\text{m}$  continues this process. In contrast, antibodies to  $\beta_2\text{m}$  bound 80 - 100% of the radiolabelled  $\beta_2\text{m}$ .

The association between  $\beta_2\text{m}$  and cod serum showed temperature-dependence. At 37°C, a high degree of binding occurred rapidly. Maximum binding was reached after 2 hours. During the first hour, quick binding, but of a lower degree, also occurred at 20°C or 40°C. After this, the binding at

20°C continued to markedly increase, whereas the binding at 40°C remained almost constant. Thus, we may be dealing with two kinds of binding, one of which is markedly influenced by the incubation temperature, the other of which is temperature-independent at the interval 4 - 20°C.

At 37°C, dissociation was considerably slower than was association. The use of anti- $\beta_2m$  antibodies in similar binding experiments gives essentially the same result. This indicates, that human  $\beta_2m$  reacts mainly with free binding sites in cod serum, rather than being bound through exchange with a corresponding cod  $\beta_2m$ -homologue. Alternatively, the  $\beta_2m$ -binding molecules have higher affinity to mammalian  $\beta_2m$  than to the presumed cod  $\beta_2m$ -homologue.

The interaction between  $\beta_2m$  and cod molecules appear to be dependent on the protein conformation of  $\beta_2m$ , of the  $\beta_2m$ -binding molecules, or of both (Fig. 6A), and seems to involve electrostatic forces (Fig. 6B). Apparently, hydrogen bonds also contribute, as the chaotropic salt NaSCN exerted inhibitory effects on the binding at lower concentrations than did NaCl or KCl (Fig. 6B). If we added 2M NaSCN after 2 hours of normal association we saw rapid dissociation, completed within 1 hour (not shown). This suggests that the  $\beta_2m$ -binding cod serum molecules and the  $\beta_2m$  associate non-covalently. The facts that the binding shows pH-optimum and temperature-dependence reminds one of enzymatic binding. On the other hand, such binding should be covalent.

The rapid inactivation of the  $\beta_2m$ -binding cod serum molecules at 50°C was unexpected. The same treatment of rat MHC Class I-antigens did not affect their ability to bind  $^{125}I$ -human  $\beta_2m$  (not shown).

The binding of  $^{125}I$ -human  $\beta_2m$  by cod serum showed specificity in comparison to irrelevant proteins, but strong cross-reactivity with  $\beta_2m$ -homologues from other mammalian species. In agreement with this, many of the data presented in this report have been reproduced using  $^{125}I$ -rat  $\beta_2m$  instead of  $^{125}I$ -human  $\beta_2m$ . Thus, the  $\beta_2m$ -binding molecules in cod serum apparently recognize a highly-conserved binding site on the various  $\beta_2m$ -homologues, but with a lower association constant for rabbit  $\beta_2m$  than for the other  $\beta_2m$ -homologues, suggesting that even this highly-conserved binding site on  $\beta_2m$  has undergone evolutionary modification.

Evolutionary studies on  $\beta_2m$  are potentially interesting. Such investigations may be performed with different experimental approaches. In previous works we have utilized the binding of heterologous  $\beta_2m$  to purified MHC class I antigens (Lögberg *et al.*, 1980), mammalian serum proteins (Lögberg *et al.*, 1981b) and various cells (Lögberg *et al.*, 1981a) in our search for evolutionally-conserved parts of the  $\beta_2m$  molecule. This approach is based on the assumption that interacting parts of  $\beta_2m$  and  $\beta_2m$ -binding molecules are of particular functional significance and thereby resistant to evolutionary pressure. The data of the present paper supports this notion and describes an inexpensive binding assay for  $\beta_2m$  which may prove useful in further studies on the evolution of this protein.

Apart from the heat-lability at 50°C, the binding of  $\beta_2m$  to cod serum has several characteristics in common with the interactions between  $\beta_2m$  and mammalian molecules. Thus the  $\beta_2m$ -binding molecules in cod serum are glycoproteins and their elution profile on Sephadex G-200 is similar to the distribution of  $\beta_2m$ -binding activity in mammalian sera (Lögberg *et al.*, 1979, 1981b). Moreover, the binding of  $\beta_2m$  by mammalian sera and

cells and by purified class I antigens, respectively, show binding constants of the same magnitude and temperature-dependence similar to that reported here (Church et al., 1982; Hyafil & Strominger, 1979; Lögdberg et al., 1981b; Lögdberg & Björck, unpublished). In mammals,  $\beta_2$ m-binding molecules, like the MHC Class I antigens, are present on the cell surface and in this paper we show binding of human  $\beta_2$ m to cod splenocytes. An interesting question raised by these data is that the  $\beta_2$ m-binding molecules in cod serum may represent homologues to corresponding mammalian  $\beta_2$ m-binding molecules.

#### ACKNOWLEDGEMENT

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# Effects of Anti-Guinea-Pig $\beta_2$ -Microglobulin Antibodies on Lymphocyte Transformation Induced by Specific Antigens or Mitogens

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Lögdberg, L., Cigén, R. & Berggård, I. Effects of Anti-Guinea-Pig  $\beta_2$ -Microglobulin Antibodies on Lymphocyte Transformation Induced by Specific Antigens or Mitogens. *Scand. J. Immunol.* 9, 263-271, 1979.

The effects of a goat anti-guinea-pig  $\beta_2$ -microglobulin antiserum ( $\alpha\beta_2m$ ) on lymphocyte transformation, induced by specific antigens or mitogens, were studied.  $\alpha\beta_2m$  was found to exert inhibitory effects on antigen stimulation, with three different antigens (purified protein derivative, hen egg-white lysozyme, and ovalbumin), and on stimulation induced by the 'T-cell mitogens', concanavalin A and phytohaemagglutinin, and the 'mixed mitogen', pokeweed mitogen. Stimulation induced by the 'B-cell mitogens', dextran sulphate and bacterial lipopolysaccharide, did not seem to be inhibited to the same extent by  $\alpha\beta_2m$ . The inhibitory effects seemed specific, as they were not seen with  $\alpha\beta_2m$  that had been absorbed on a column with insolubilized purified guinea-pig  $\beta_2m$  nor with normal goat serum. Time studies indicated that the inhibitions started already during the first day of culture.

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Human  $\beta_2$ -microglobulin ( $\beta_2m$ ) [4] is a small protein, found both free in various body fluids [4] and bound to cell surfaces [19, 20]. It has attracted the interest of immunologists because of its sequence homology with constant immunoglobulin domains [19, 23] and its physical association, on both the cell surface and in solution, to the strong transplantation antigens (HLA) [9]. Furthermore, in the mouse, other molecules, structurally related to the strong transplantation antigens, also seem to contain mouse  $\beta_2m$  [1, 14, 16, 18, 27].

$\beta_2m$  appears to be of importance in the activation of human lymphocytes, as antibodies against human  $\beta_2m$  can impede the response in mixed lymphocyte reactions (MLR) [2, 11-13, 17, 24, 24-26] and in antigen- [12, 17, 24-26] or mitogen- [3, 13, 17, 24, 26] induced lymphocyte transformation. On this basis, it has been suggested that  $\beta_2m$  could be a part of receptors for antigen, mitogen, and MLR on the lymphocyte cell surface. Antibodies against human  $\beta_2m$  also activate B-lymphocytes from

man and mouse [15, 22, 25]. Therefore antibodies against  $\beta_2m$  should be important tools in the study of lymphocyte activation.

Recently, the guinea-pig homologue of  $\beta_2m$  was purified in one of our laboratories [8]. This paper describes some effects of antibodies against this protein in lymphocyte transformation tests. It has previously been shown with the same antibodies that the association of  $\beta_2m$  to the strong transplantation antigens can be extended to the guinea-pig system [6].

## MATERIALS AND METHODS

**Animals.** Random bred guinea-pigs, weighing 300-350 g, were obtained from Sahlin, Malmö, Sweden.

**Antiserum and IgG.** A goat antiserum was prepared against purified guinea-pig  $\beta_2m$  [6]. Serum from the same bleeding was used throughout this investigation. The IgG fraction was prepared by ion exchange chromatography of the antiserum on DEAE-cellulose 23-SH (Serva Feinbiochemia, Heidelberg, West Germany) in 0.02 M Tris-HCl buffer, pH 7.8, using linear salt gradient elution. The material eluting with NaCl

† Deceased June 1978.

concentrations from 0 to 0.06 M was saved and further purified by chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden). Both the antiserum and the IgG fraction, preserved with 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, were dialysed exhaustively against phosphate-buffered saline (PBS), inactivated for 30 min at 56°C, and passed through a 0.22 µm filter (Millipore, Bedford, Mass., USA) before being used in the culture systems. To check the specificity of the effects of the antiserum, a sample of it was absorbed on a column with cyanogen bromide (CNBr)-Sephadex coupled guinea-pig β<sub>2</sub>m. The absorbed antiserum (aβ<sub>2</sub>m<sub>abs</sub>) contained less than 0.1% specific anti-guinea-pig β<sub>2</sub>m antibodies compared with unabsorbed antiserum by radioimmunoassay. The titre of precipitating antibodies in the unabsorbed antiserum was 0.1 mg/ml, measured by double radial immunodiffusion.

**Antigens.** Purified protein derivative (PPD) (Statens Seruminstitut, Copenhagen, Denmark), hen egg-white lysozyme (Boehringer-Mannheim, West Germany), and ovalbumin (Sigma, St Louis, Mo., USA) were used from stock solutions of 1 mg/ml in saline.

**Mitogens.** Phytohaemagglutinin (PHA)-M and -P (Difco, Detroit, Mich., USA), concanavalin A (Con A) (Sigma), pokeweed mitogen (PWM) (Sigma), lipopolysaccharide (LPS) 055:B5 (Sigma), and dextran sulphate (DxS) (MW ~ 500,000, sulphur content 17 ± 1%) (Pharmacia) were used at various concentrations.

**Immunization.** Guinea-pigs were injected in all four paws with antigenic solutions (1 mg/ml) or 0.9% saline emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco). In each paw, 0.05 ml of the emulsion was injected into one toe pad. The animals were used 10–16 days after immunization.

**Cell sources.** Regional lymph nodes from immunized animals or spleens from immunized or untreated animals were extirpated under aseptic conditions, washed and trimmed in Parker 199 medium (SBL, Stockholm, Sweden), and then teased in fresh Parker 199. The cell suspension thus made was filtered through double gauze to give a single cell suspension.

**Cell cultures.** Cells were cultured in autoclaved, disposable plastic tubes (GBR Instruments, Hvidovre, Denmark), each tube containing 1 ml medium, composed of 20% heat-inactivated horse serum (Flow Laboratories, Ayrshire, Scotland) and 80% Parker 199 with antibiotics added, and  $1.5 \times 10^6$  lymph node cells or  $2 \times 10^6$  spleen leucocytes. Antigens, mitogens and anti-guinea-pig β<sub>2</sub>m were diluted with saline and used in the concentrations stated below. Control cultures received only saline. Three parallel cultures were always prepared. The cultures in air atmosphere were closed with re-used rubber stoppers and incubated at 37°C for 3 days, unless otherwise stated.

**Assay of DNA synthesis.** At the end of the culture period, tritiated thymidine (methyl-<sup>3</sup>H-thymidine, Schwarz/Mann, Orangeburg, USA, 1.9 Ci/mm) was added to a final concentration of 1 µCi/ml. After 1 h of incubation with the isotope, the cells were spun down, washed once with phosphate buffer, pH 7.2, extracted for nucleosides and nucleotides for 30 min with cold trichloroacetic acid (TCA), and then washed once with TCA and once with absolute ethanol. The residue was dissolved in 0.5 ml Soluene 350 (Packard Ltd, Downers Grove, USA) and mixed with 2.5 ml Insta-

Fluor (Packard Ltd). Radioactivity was assayed in a Packard Tri-Carb 3310 liquid scintillator for a 10 min counting. The whole procedure, including the liquid scintillation, was carried out in the culture tubes.

**Statistical considerations.** In order to obtain a near-normal distribution of the variates, recorded cpm values were transformed to log<sub>10</sub> values [10]. Effects were evaluated as the log<sub>10</sub> of stimulation indices: log<sub>10</sub> SI = mean log<sub>10</sub> cpm of stimulated cultures minus mean log<sub>10</sub> cpm of control cultures. The inhibitory activity of anti-guinea-pig β<sub>2</sub>m (aβ<sub>2</sub>m) was evaluated as the difference between log<sub>10</sub> SI with aβ<sub>2</sub>m and log<sub>10</sub> SI without aβ<sub>2</sub>m. In order to estimate significance limits for 'effects' and 'inhibitory activities on effects', *t* tests were performed on the basis of the technical error as determined from a number of 'identically' performed cultures. The standard deviation varied between culture systems. Lymph node cells cultured for 3 days gave an SD value of 0.126 (806 df). The corresponding values for the spleen cell systems were 0.0779 (240 df), 0.110 (1250 df) and 0.147 (80 df) for 2, 3 and 5 days of culture, respectively.

When two 'identical' triplets are compared in the spleen cell system cultured for 3 days, a random difference in mean log<sub>10</sub> cpm values of 0.23 (less than a doubling for cpm values) will occur only once in a hundred experiments (two-tailed *t* test). Only once in a hundred experiments would an inhibitory effect of 0.33 on a log<sub>10</sub> SI value arise by chance (one-tailed *t* test).

## RESULTS

### *Effect of aβ<sub>2</sub>m on background level*

aβ<sub>2</sub>m was rarely found to exert any significant effect on lymph node cells (Fig. 1A), but such an effect was the rule rather than the exception on spleen cells (Fig. 1B), on 2-, 3- and 5-day cultures (the figure shows only 3-day cultures). Therefore, in the spleen cell systems, the effects with aβ<sub>2</sub>m were compared both with those without aβ<sub>2</sub>m (aβ<sub>2</sub>m-effect on background expected to be multiplicative) and with those calculated with the effect of aβ<sub>2</sub>m on background expected to be additive to that of the different mitogens (see Table II).

### *Effect of aβ<sub>2</sub>m on antigen stimulation of lymph node cells*

First, the ability of aβ<sub>2</sub>m to impede PPD stimulation of CFA-activated regional lymph node cells was investigated. Fig. 2A shows the results. In all cases when a significant effect of PPD was measured, a corresponding significant inhibition of this effect was seen when aβ<sub>2</sub>m was added. The antiserum was as inhibitory when

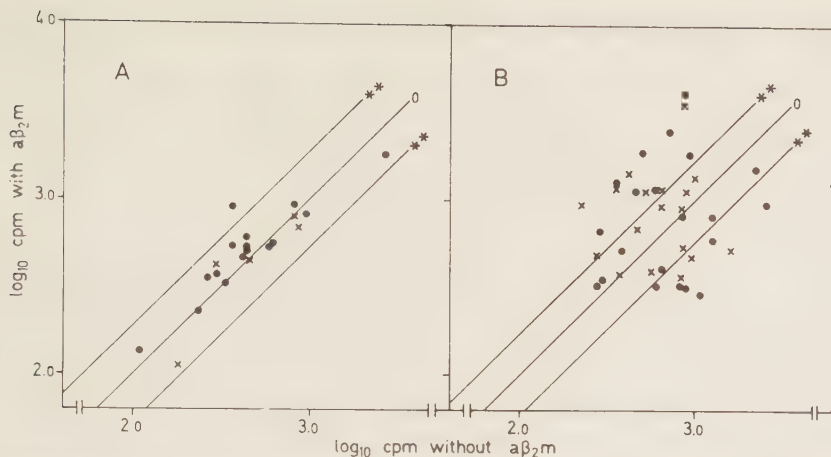


FIG. 1.  $^3H$ -thymidine uptake in cultures with  $a\beta_2m$  plotted against uptake in control cultures. (A) Lymph node cells cultured 3 days. (B) Spleen cells cultured 3 days. The significance limits at the 1% level (\*\*) are given.  $a\beta_2m$  dilution 1:20 (x) or 1:100 (●).

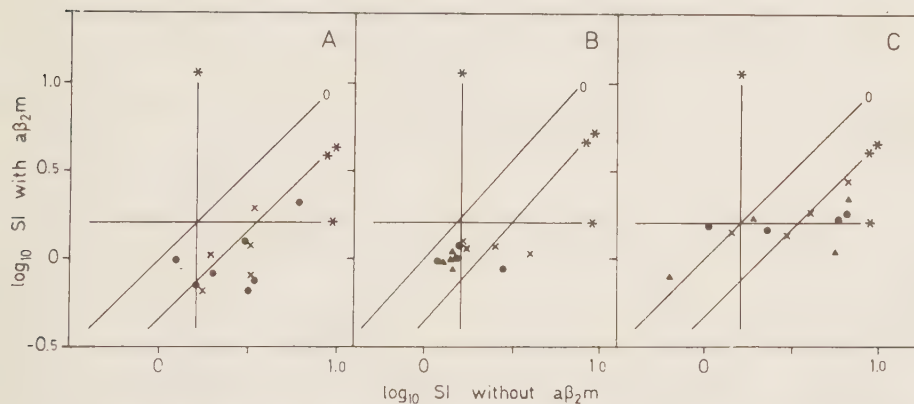


FIG. 2. Antigen stimulation of lymph node cells in the presence of  $a\beta_2m$  plotted against antigen stimulation without  $a\beta_2m$ . (A) Purified protein derivative (PPD) stimulation,  $a\beta_2m$  1:20 (x) or 1:100 (●). PPD concentration, 50 or 10  $\mu g/ml$ . (B) Hen egg-white lysozyme stimulation,  $a\beta_2m$  1:100. Lysozyme concentrations, 50  $\mu g/ml$  (x), 10  $\mu g/ml$  (●) and 2  $\mu g/ml$  (▲). (C) Ovalbumin stimulation,  $a\beta_2m$  1:100. Ovalbumin concentrations, 50  $\mu g/ml$  (x), 10  $\mu g/ml$  (●) and 2  $\mu g/ml$  (▲). Significance limits for antigen effects at the 5% level (\*) and for inhibitory activity of  $a\beta_2m$  at the 1% level (\*\*) are given.

ed in dilution 1:100 as in dilution 1:20. accordingly, the former dose was used in most of the subsequent experiments. The dilutions 1:500 and 1:2500 were not inhibitory.

**Generalization to other antigens.** Four animals were immunized with hen egg-white lysozyme and four with ovalbumin. The regional lymph node cells were stimulated with three doses of the corresponding antigen with or without the presence of  $a\beta_2m$ . The results are combined in

Fig. 2B (lysozyme) and Fig. 2C (ovalbumin). All the effects of lysozyme were inhibited by  $a\beta_2m$ , but as the effects were in many instances very weak, only a few  $a\beta_2m$ -induced inhibitions reached significance level. In three of the four animals immunized with ovalbumin, all doses gave significant stimulations, all being inhibited by  $a\beta_2m$ ; in most cases the inhibitions were significant. When antigens were tested at the two higher concentrations, on lymph node cells from animals immunized only with CFA/saline,



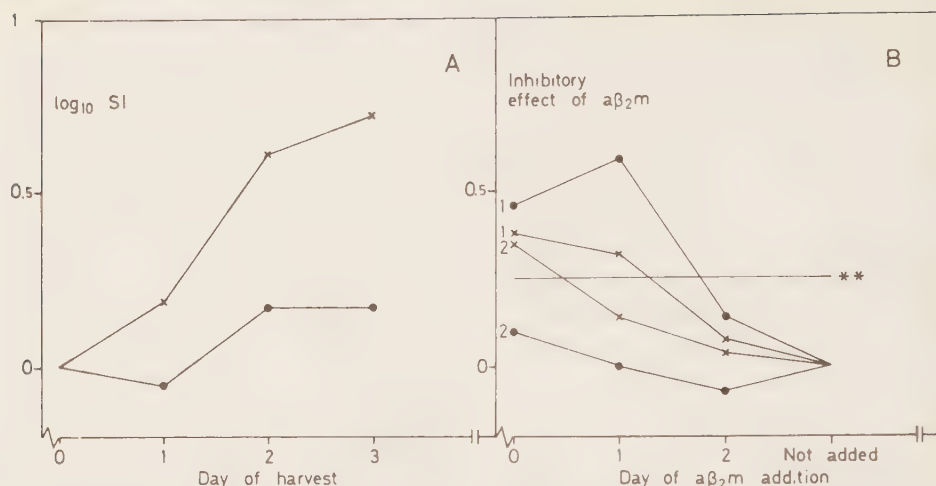


FIG. 3. Kinetic studies of the  $\alpha\beta_2\text{m}$ -induced inhibitions of purified protein derivative (PPD) stimulation of lymph node cells. (A) PPD stimulation (50 µg/ml) on different days after initiation, with (●) or without (×)  $\alpha\beta_2\text{m}$  IgG, 0.5 mg/ml, present in the cultures. (B) Inhibitory effect of  $\alpha\beta_2\text{m}$  1:100 on PPD stimulation, when  $\alpha\beta_2\text{m}$  was added at different times after culture initiation. PPD concentrations, 50 µg/ml (×) and 10 µg/ml (●). Results from two different experiments are shown. The significance limit for inhibitory activity of  $\alpha\beta_2\text{m}$  at the 1% level (\*\*) is given in (B).

TABLE I. Control of specificity of the  $\alpha\beta_2\text{m}$ -induced inhibition of purified protein derivative (PPD) stimulation of lymph node cells and phytohaemagglutinin (PHA) stimulation of spleen cells\*

| Stimulus        | $\log_{10}$ SI | Effect on stimulation by addition of: |                                           |          |          |
|-----------------|----------------|---------------------------------------|-------------------------------------------|----------|----------|
|                 |                | $\alpha\beta_2\text{m}$ 1:20          | $\alpha\beta_2\text{m}_{\text{abs}}$ 1:20 | NGS 1:20 | PBS 1:20 |
| PPD (50 µg/ml)  | 0.51           | -0.60***                              | -0.20 NS                                  | ±0 NS    | -0.04 NS |
| PPD (5 µg/ml)   | 0.29           | -0.37**                               | +0.06 NS                                  | -0.05 NS | -0.04 NS |
| PHA (100 µl/ml) | -0.03          | -0.74***                              | -0.12 NS                                  | -0.08 NS | -0.03 NS |
| PHA (5 µl/ml)   | 0.73           | -0.50***                              | -0.05 NS                                  | -0.04 NS | +0.06 NS |
| PHA (0.5 µl/ml) | 0.40           | -0.55***                              | -0.07 NS                                  | -0.07 NS | +0.01 NS |

NGS = normal goat serum; PBS = phosphate-buffered saline.

\* The deviations from zero effect on the various stimulations exerted by the additions were tested for statistical significance as described in Materials and Methods. NS = not significant,  $P > 0.05$ ; \*\* =  $0.01 > P > 0.001$ ; \*\*\* =  $0.001 > P$ .

no significant stimulations occurred. The average effects (mean  $\log_{10}$  SI  $\pm$  SEM) were  $-0.05 \pm 0.04$  and  $-0.13 \pm 0.12$  with 50 µg antigen/ml and  $-0.05 \pm 0.07$  and  $-0.02 \pm 0.08$  with 10 µg antigen/ml for lysozyme (six experiments) and ovalbumin (three experiments), respectively.

**Kinetics.** When cultures were harvested on different days, it could be seen that the inhibitory effect of  $\alpha\beta_2\text{m}$  on PPD stimulation had started already during the first day of culture, as demonstrated in Fig. 3A. When  $\alpha\beta_2\text{m}$  was added at different times after the initiation of the

antigen stimulation, results as in Fig. 3B were obtained. A 24 h delay before addition still allowed  $\alpha\beta_2\text{m}$  to be inhibitory.

**Control of specificity.** The inhibitory effect of  $\alpha\beta_2\text{m}$  was simultaneously compared with the effects of PBS, normal goat serum (NGS), and a sample of  $\alpha\beta_2\text{m}$  that had been absorbed on a column of insolubilized purified guinea-pig  $\beta_2\text{m}$ . Table I shows the result of such an experiment.  $\alpha\beta_2\text{m}$  significantly inhibited PPD stimulation at two dose levels of PPD. But the other three agents did not significantly alter the

TABLE II. Average mitogen effects on spleen cells with or without the presence of a goat anti-guinea-pig  $\beta_2$ -microglobulin serum\*

|                                     | $a\beta_2m$ -<br>dilution | (a)<br>Average<br>$\log_{10}$ SI<br>without $a\beta_2m$ | (b)<br>Average<br>$\log_{10}$ SI if<br>$a\beta_2m$ effect<br>on background<br>is added | (c)<br>Average<br>$\log_{10}$ SI<br>with $a\beta_2m$ | No. of<br>guinea-pigs<br>examined |
|-------------------------------------|---------------------------|---------------------------------------------------------|----------------------------------------------------------------------------------------|------------------------------------------------------|-----------------------------------|
| Concanavalin A                      |                           |                                                         |                                                                                        |                                                      |                                   |
| 2–25 $\mu g/ml$ sub-<br>optimum     | 1:20                      | 0.53 $\pm$ 0.13                                         | 0.41 $\pm$ 0.09                                                                        | -0.02 $\pm$ 0.06                                     | 11                                |
| 12.5–50 $\mu g/ml$<br>optimum       | 1:20                      | 1.04 $\pm$ 0.16                                         | 0.91 $\pm$ 0.14                                                                        | 0.22 $\pm$ 0.16                                      | 9                                 |
| 25–250 $\mu g/ml$ supra-<br>optimum | 1:20                      | 0.65 $\pm$ 0.22                                         | 0.59 $\pm$ 0.19                                                                        | -0.10 $\pm$ 0.22                                     | 6                                 |
| Phytohaemagglutinin                 |                           |                                                         |                                                                                        |                                                      |                                   |
| 0.2–1 $\mu l/ml$ sub-<br>optimum    | 1:20                      | 0.40 $\pm$ 0.11                                         | 0.31 $\pm$ 0.11                                                                        | -0.08 $\pm$ 0.07                                     | 6                                 |
| 5 $\mu l/ml$ optimum                | 1:20                      | 0.79 $\pm$ 0.10                                         | 0.79 $\pm$ 0.09                                                                        | 0.26 $\pm$ 0.09                                      | 7                                 |
| 100 $\mu l/ml$ supra-<br>optimum    | 1:20                      | -0.12 $\pm$ 0.07                                        | -0.17 $\pm$ 0.10                                                                       | -0.64 $\pm$ 0.15                                     | 6                                 |
| Dextran sulphate                    |                           |                                                         |                                                                                        |                                                      |                                   |
| 1 mg/ml                             | 1:100                     | 0.41 $\pm$ 0.08                                         | 0.48 $\pm$ 0.05                                                                        | 0.61 $\pm$ 0.15                                      | 5                                 |
| Lipopolysaccharide                  |                           |                                                         |                                                                                        |                                                      |                                   |
| 500 $\mu g/ml$                      | 1:100                     | 0.34 $\pm$ 0.07                                         | 0.48 $\pm$ 0.10                                                                        | 0.29 $\pm$ 0.11                                      | 6                                 |
| Pokeweed mitogen                    |                           |                                                         |                                                                                        |                                                      |                                   |
| 0.5 $\mu g/ml$                      | 1:100                     | 0.80 $\pm$ 0.07                                         | 0.44 $\pm$ 0.12                                                                        | 0.18 $\pm$ 0.30                                      | 4                                 |
| 0.05 $\mu g/ml$                     | 1:100                     | 0.49 $\pm$ 0.15                                         | 0.23 $\pm$ 0.08                                                                        | 0.08 $\pm$ 0.39                                      | 3                                 |

\* Values are means  $\pm$  SEM. If  $a\beta_2m$ 's effect on background is multiplicative to the mitogen effect, the net effect of  $a\beta_2m$  on the mitogen effect is calculated as column c - column a. If the two effects are additive, the latter expression will be column c - column b.

response. The purified IgG fraction also inhibited PPD stimulation. The time study in Fig. 3A was achieved with IgG purified from the  $a\beta_2m$  antiserum.

#### *Effect of $a\beta_2m$ on mitogen stimulation of spleen cells*

Table II summarizes these studies.

#### *Effect on stimulation with 'T-cell mitogens'*

We used Con A and PHA-M as 'T-cell mitogens'. It is evident from Fig. 4A–B and Table II that the stimulation with both these mitogens was effectively inhibited by  $a\beta_2m$  at a dilution of 1:20. The stimulations with suboptimum doses of mitogen were generally almost completely eliminated by addition of  $a\beta_2m$ . This is in contrast to the stimulations with optimum mitogen doses, where a small residual stimulation usually persisted after the addition of  $a\beta_2m$ . With supra-

optimum doses, the strength of the mitogen stimulations decreased, following the traditional clock form of the dose-response curve. None the less, the inhibitory capacity of  $a\beta_2m$  remained similar to the effect on optimum stimulations. This means that the combination of supraoptimum mitogen doses and  $a\beta_2m$  sometimes led to relatively strong inhibitions of the  $^3H$ -thymidine uptake compared with cultures with only  $a\beta_2m$ . From dose-response curves and accumulated data, we concluded that  $a\beta_2m$  in the dilution 1:100 had a similar inhibitory capacity on the PHA stimulations as the 1:20 dilution. The former dose was therefore used in most of the following experiments. The dilutions 1:500 and 1:2500 were not inhibitory. Stimulations induced by PHA-P were also inhibited by  $a\beta_2m$ .

*Kinetics.* When cultures were harvested on different days, it could be seen that the inhibition of PHA stimulation caused by  $a\beta_2m$  was

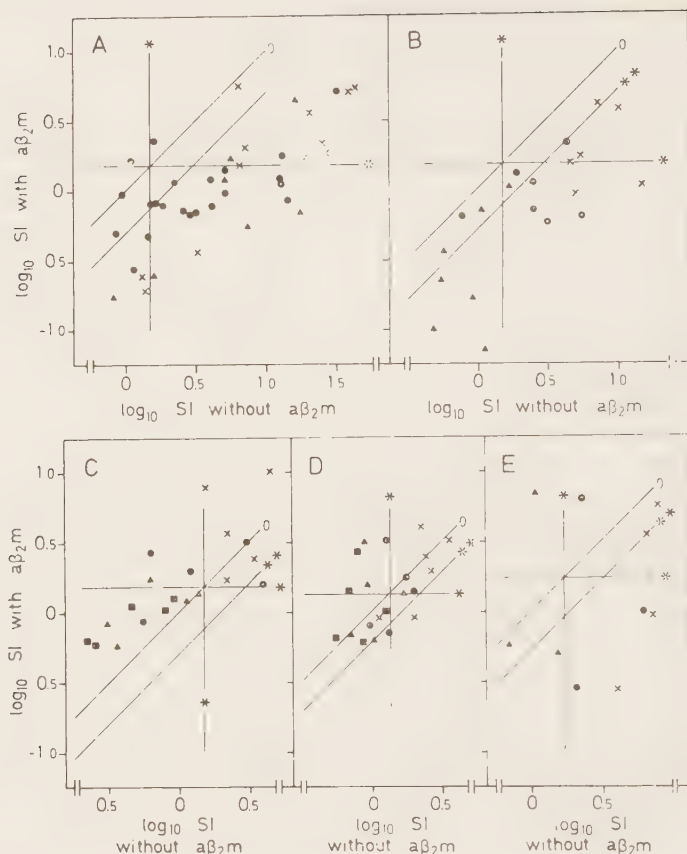


FIG. 4. Mitogen stimulation of spleen cells in the presence of  $a\beta_2m$ , plotted against mitogen stimulation without  $a\beta_2m$  ( $a\beta_2m$  effect on background supposed to be multiplicative). (A) Concanavalin A stimulation. Suboptimum (●), optimum (×) and supraoptimum (▲) Con A concentrations were used.  $a\beta_2m$  dilution, 1:20. (B) Phytohaemagglutinin (PHA) stimulation. Suboptimum (●), optimum (×) and supraoptimum (▲) PHA concentrations were used.  $a\beta_2m$  dilution, 1:20. (C) Dextran sulphate (DxS) stimulation. DxS concentrations, 1 mg/ml (×), 0.1 mg/ml (●), 0.01 mg/ml (▲) and 0.001 mg/ml (■).  $a\beta_2m$  dilution, 1:100. (D) Lipopolysaccharide (LPS) stimulation. LPS concentrations, 500  $\mu\text{g/ml}$  (×), 150  $\mu\text{g/ml}$  (●), 50  $\mu\text{g/ml}$  (▲) and 15  $\mu\text{g/ml}$  (■).  $a\beta_2m$  dilution, 1:100. (E) Pokeweed mitogen (PWM) stimulation. PWM concentrations, 500 ng/ml (×), 50 ng/ml (●) and 5 ng/ml (▲).  $a\beta_2m$  dilution 1:20 or 1:100. Significance limits for mitogen effects at the 5% level (\*) and for inhibitory activity of  $a\beta_2m$  at the 1% level (\*\*) are given.

initiated already during the first day of culture. When  $a\beta_2m$  was added at different times after the beginning of the PHA stimulation, it was apparent that a 48 h delay before addition still allowed  $a\beta_2m$  to exert some inhibitory effect. The overall picture was very similar to the time studies on antigen stimulation (see Fig. 3).

**Control of specificity.** The specificity of the inhibitions was checked in the same way as in

the study of antigen stimulation. Table I shows an example of such an experiment. On stimulation with three PHA doses, one suboptimum, one optimum, and one supraoptimum,  $a\beta_2m$  gave highly significant inhibitions. PBS, NGS and absorbed  $a\beta_2m$  had no effect. The inhibitions of Con A stimulations were specific in the same manner. Moreover, the IgG fraction of the antiserum was inhibitory for both PHA and Con A stimulations.

*Effect on stimulation with 'B-cell mitogens'*

Spleen cells from five guinea-pigs were stimulated with four doses of DxS, and spleen cells from five other guinea-pigs were stimulated with four doses of LPS. Spleen cells from an additional animal were stimulated only with the highest dose of LPS. The effect of  $\alpha\beta_2m$ , 1:100, was tested in all experiments, and the results were combined in Fig. 4C (DxS) and Fig. 4D (LPS). Only the highest doses (1 mg DxS/ml and 500  $\mu$ g LPS/ml) gave reproducible and significant stimulations of the same magnitude as suboptimum doses of the 'T-cell mitogens'. Instead of being completely abolished by  $\alpha\beta_2m$ , like the 'T-cell mitogen' stimulations, these stimulations were rather slightly potentiated (DxS) or only weakly inhibited (LPS). It should be noted that cells stimulated with LPS were only cultured for 2 days.

*Effect on stimulation with a 'mixed mitogen'*

Spleen cells from three guinea-pigs were stimulated with three doses of PWM and spleen cells from an additional animal only with the highest dose, and the effects of  $\alpha\beta_2m$ , 1:20 or 1:100, were investigated in each experiment. The results are shown in Fig. 4E. The cells were cultured for 5 days. There is a rather high variation in the results, but the ability of  $\alpha\beta_2m$  to impede the PWM response is evident. Similar to the 'T-cell mitogens',  $\alpha\beta_2m$  1:20 and 1:100 were inhibitory, in contrast to 1:500 and 1:2500. When cultures were harvested on days 1, 3 and 5 it was apparent that  $\alpha\beta_2m$ -caused inhibitions had already begun during the first day of culture.

*Effect of  $\alpha\beta_2m$  on mitogen stimulation of lymph node cells*

These studies were performed with Con A, PHA-M and PWM as mitogens. The stimulations induced by the mitogens were much higher than the corresponding stimulations seen on spleen cells. The mitogen effects were inhibited by  $\alpha\beta_2m$ , especially when suboptimum mitogen doses were used. Only very weak inhibitions were seen if optimum or supraoptimum doses were used. The typical pattern for PHA stimulation is shown in Fig. 5.

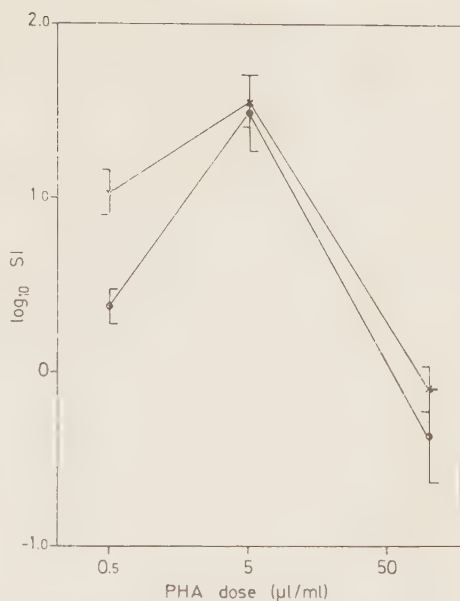


FIG. 5. Phytohaemagglutinin (PHA) stimulation of lymph node cells with (●) or without (×)  $\alpha\beta_2m$  1:20 or 1:100 (mean  $\pm$  SEM, four experiments).

## DISCUSSION

The present work has clearly established that  $\alpha\beta_2m$  is able to impede the cellular response to different specific antigens and to different 'T-cell mitogens' in lymphocyte transformation experiments. But the response to 'B-cell mitogens' was not inhibited to the same extent. In the case of DxS stimulation, it was rather potentiated by  $\alpha\beta_2m$ . The inhibitory capacity of the  $\alpha\beta_2m$  antiserum appeared to be specifically exerted by antibodies against guinea-pig  $\beta_2m$ .

The finding of differences in inhibiting activity when different mitogen concentrations were used on spleen or lymph node cells is not opposed to the hypothesis that  $\beta_2m$  is a part of mitogen receptors on T cells. Thus, the fact that stimulation of spleen cells is not completely abolished by the addition of  $\alpha\beta_2m$  at optimum doses, but at suboptimum doses of mitogen, could be explained by a model in which  $\alpha\beta_2m$  and the different 'T-cell mitogens' compete for the mitogen receptors. When the mitogen dose is increased, the  $\alpha\beta_2m$  dose is no longer sufficient for complete blockade. It is conceivable

that higher concentrations of  $\alpha\beta_2m$  could completely eliminate the optimum mitogen effects. This, owing to shortage of the  $\alpha\beta_2m$ , has not been tested. Similarly, the differences between the  $\alpha\beta_2m$ -induced inhibitions of lymph node cell responses, when suboptimum or optimum mitogen doses were used, could be explained by this model. The differences between the inhibitions of spleen and lymph node cell responses could indicate a higher density of mitogen receptors on lymph node T cells, or perhaps it only reflects a higher percentage of T cells in the lymph nodes than in the spleen. With supraoptimum mitogen doses, the  $\log_{10}$  SI values of the stimulations decreased. This is not an actual decrease of stimulation but rather a masking of the effect by a simultaneous cytotoxic effect of the high mitogen concentrations [5]. This can explain the strong inhibitory effects sometimes exerted by  $\alpha\beta_2m$  in combination with supraoptimum PHA doses. By inhibiting the masked stimulatory effect of the supraoptimum PHA dose,  $\alpha\beta_2m$  reveals the cytotoxic PHA activity. The same line of argument could be used to explain these differences as quantitative in nature even if  $\alpha\beta_2m$  is supposed to impede the mitogen stimulations at a later step than mitogen binding. A similar dependence of mitogen concentration on the ability to impede the response with  $\alpha\beta_2m$  has been noted in the human system [3, 24, 26] and could explain why some authors find only weak [12, 17] effects or none at all [11] of  $\alpha\beta_2m$  on mitogen stimulation.

$\alpha\beta_2m$ -induced inhibition of antigen stimulation has been demonstrated in the human system only for PPD as antigen [12, 17, 24–26]. In this study we extend this finding to two other antigens. Antigen-induced lymphocyte transformation is thought to be predominantly a T-cell effect. Thus, it seems as though  $\alpha\beta_2m$  can impede the activation of T lymphocytes but not, or less effectively, of B lymphocytes. Consistent with this,  $\alpha\beta_2m$  has been shown to delay the rejection of grafted hearts in the rat system [7].

The results of the time studies tell us that  $\alpha\beta_2m$  acts on a relatively early step in the lymphocyte activation, perhaps by steric blockade of the antigen and mitogen receptors. It is also conceivable that it is some intermediate step which is inhibited, and it is tempting to suggest that  $\alpha\beta_2m$  might act by suppression of a common pathway in the T-cell activation. One such point of action could be on recruiting lympho-

kines (in this case blastogenic factors for T cells).  $\alpha\beta_2m$  can absorb at least two cell-cell interaction factors: the allogeneic effect factor [1] and the human granulocyte-colony-stimulating factor [21].

Concerning the effect of  $\alpha\beta_2m$  on background thymidine uptake, we saw few effects on lymph node cells under the conditions used. But on spleen cells, both significant stimulations and inhibitions occurred with high frequency. This made a double comparison necessary to assess the inhibitory capacity of  $\alpha\beta_2m$ ; the  $\alpha\beta_2m$  effect on background was either expected to be additive or multiplicative to the mitogen effects. In many reports on the effect of  $\alpha\beta_2m$  in human systems, it is not clear whether proper consideration has been given to the effects of  $\alpha\beta_2m$  on background activity. Our time studies indicated that sometimes the horse serum used to support the medium could provoke a proliferative response in spleen cells. The inhibitory effects of  $\alpha\beta_2m$  on background uptake could then be regarded as an inhibition of antigen stimulation. The background inhibition might alternatively be explained by suppressor cell activation.

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# VII

## $\beta_2$ -MICROGLOBULIN - ACCEPTOR FOR THE LYTIC SIGNAL FROM NATURAL KILLER CELLS?

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Natural killer (NK) cells are a minor population of lymphocyte-like cells that are functionally defined by their ability to destroy certain tumor, virus-infected, or normal cells without apparent prior sensitization<sup>1</sup>. They are currently believed to constitute the major frontline defence in tumor surveillance. The molecular mechanisms involved in target binding and lysis are still not clearly understood. The lytic phase of NK cell-mediated killing has recently been reported to involve lymphotoxin (LT)-like factor(-s)<sup>2,3</sup>. Since "innocent bystanders" are not killed during NK-target cell interaction and the lytic event is completed far more rapidly than by soluble LT, an acceptor site able to focus LT on the target cell has been postulated<sup>4</sup>. We have investigated whether antibodies directed against the well-characterized cell surface protein  $\beta_2$ -microglobulin ( $\beta_2m$ )<sup>5</sup> can affect the NK-target cell interaction. Anti- $\beta_2m$  specifically inhibited basal as well as interferon (IFN)-activated NK cell-mediated cytotoxicity. This effect depended on the binding of anti- $\beta_2m$  to target cells and was specific for the lytic phase, i.e., formation of NK-target cell conjugates was not affected. These data can be interpreted to mean that  $\beta_2m$  (or a  $\beta_2m$ -associated structure) fulfills the role as acceptor for the lytic signal from NK cells to their target cells.

The effect of anti- $\beta_2m$  on human NK-activity was first tested by continuous presence of the antibodies during the cytotoxicity assay. Table 1 summarizes these results. Goat anti-human  $\beta_2m$  (GaHu $\beta_2m$ ) serum (1:20) clearly impeded the cytotoxic effect of human peripheral blood leukocytes (PBL) against K562-cells (58% inhibition), whereas the same concentration of normal goat serum (NGS) only led to marginal reduction of the NK-activity (8% inhibition). A goat antiserum against the blood group antigen Lewis<sup>b</sup> (Ortho Pharmaceuticals, New Jersey), which stained K562 cells in indirect immunofluorescence, gave the same weak effects as NGS. The purified IgG, instead of whole sera, gave similar results. Furthermore, F(ab')<sub>2</sub>- or Fab'-fragments of the anti- $\beta_2m$ -IgG could almost completely block the cytotoxic reaction (up to 90% inhibition). This rules out the possibility that the inhibitory effect involves Fc-receptor-based mechanisms. On the other hand, the Fc-receptors are implicated as the explanation for the lack of complete inhibition by anti- $\beta_2m$  serum or IgG. Thus, in these cases, the inhibitory effect may be partly masked by simultaneous antibody-dependent cell-mediated cytotoxicity (ADCC). When anti- $\beta_2m$  serum was absorbed on a column of human  $\beta_2m$ -Sephadex, it completely lost its inhibitory capacity. Anti- $\beta_2m$ -activity recovered from this column by low-pH elution exhibited the same degree of inhibition of NK-activity as did whole anti- $\beta_2m$  serum. This demonstrates the specificity for  $\beta_2m$  of the effect observed. Similarly, addition of excess amounts of purified human  $\beta_2m$  to the NK-assay completely reversed the inhibitory action of the anti- $\beta_2m$  antibodies.

The inhibitory effect of anti- $\beta_2m$  was not restricted to K562, but could be also demonstrated with other cell types as target cells; two T-cell-lines (CCRF-CEM or SKW-3) and a fibroblast primary culture (S199N-T2) (see Table 1). The Table shows experiments performed with an effector:target cell ratio of 50:1. A similar effect of anti- $\beta_2m$  was seen also at other effector:target cell ratios. Dose-response experiments revealed significant inhibition of NK-activity by anti- $\beta_2m$  serum to a dilution of 1:2,560. A rabbit anti-human  $\beta_2m$  serum was an even more potent inhibitor of NK-activity than the GaHu $\beta_2m$  serum (data not shown).

To investigate whether anti- $\beta_2m$  is able to interfere not only with basal NK-mediated killing, but also with the cytotoxicity of IFN-activated NK cells, PBLs were preincubated with IFN (10<sup>7</sup> cells and 250 IU IFN/ml;

**Table 1** Effects of anti- $\beta_2$ -microglobulin on natural killer cell-mediated cytotoxicity against different target cells

| Target cells                      | K562                                                       | CCRF-CEM      | SKW-3          | S199N-T2      |
|-----------------------------------|------------------------------------------------------------|---------------|----------------|---------------|
| Inhibitor                         | % inhibition of $^{51}\text{Cr}$ -release: mean (range; n) |               |                |               |
| NGS 1:20                          | 8 ( 0-21; 4)                                               | ND            | ND             | ND            |
| NG-IgG (0.75 mg/ml)               | 5 ( 0-14; 14)                                              | 10 ( 3-18; 4) | 9 ( 5-12; 3)   | 4 ( 0-8; 3)   |
| GaHu $\beta_2$ m - serum 1:20     | 58 (44-71; 4)                                              | ND            | ND             | ND            |
| - IgG (0.75 mg/ml)                | 64 (40-86; 14)                                             | 60 (51-68; 4) | 73 (55-100; 3) | 66 (49-70; 3) |
| - F(ab') <sub>2</sub> (0.5 mg/ml) | 88 (80-96; 5)                                              | 83 (81-85; 2) | 90 (87-93; 2)  | 81 (76-86; 2) |
| - F(ab') (0.5 mg/ml)              | 90 (83-95; 4)                                              | ND            | ND             | ND            |
| - serum                           |                                                            |               |                |               |
| absorbed 1:20                     | 7 ( 0-16; 3)                                               | 11 ( 8-14; 2) | ND             | ND            |
| - antibodies                      |                                                            |               |                |               |
| affinity purified                 | 65 (58-69; 3)                                              | 66 (65-67; 2) | ND             | ND            |
| - IgG + Hu $\beta_2$ m            | 14 (10-18; 2)                                              | ND            | ND             | ND            |

Mononuclear cells (PBL) were separated from peripheral blood of normal healthy donors by flotation on Lymphoprep<sup>R</sup> (Nyegaard, Oslo, Norway). PBL were used as effector cells in a 4 hour <sup>51</sup>Cr-release assay as described<sup>24</sup>. The NK nature of the effector cells is inferred from an 80 % reduction in activity after treatment with the monoclonal anti-human NK antibody HNK-1 (Leu 7, Becton-Dickinson, Ca.)<sup>25</sup> and rabbit complement, and the lack of effect when monoclonal OKT 3 antibodies (Ortho Pharmaceuticals, New Jersey) were included in the assay. The latter antibody is known to abrogate T-cell-mediated cytotoxicity<sup>26</sup>. The targets cells were K562 (erythroleukemic line)<sup>27</sup>, CCRF-CEM (T-cell line)<sup>28</sup>, SKW-3 (T-cell line)<sup>29</sup>, and S199N-T2 (primary fibroblast culture). S199N-T2 cells were cultured as monolayer in RPMI 1640 medium supplemented with 10 % fetal calf serum and penicillin-streptomycin (complete medium); the other cell lines were maintained as stationary suspension cultures in the same medium. The NK-assay was performed by co-culturing 10<sup>4</sup> target cells in complete medium with various numbers of effector cells in the presence or absence of the substance to be tested. Only data from effector:target cell ratio 50:1 are shown. Percent inhibition is related to assay in medium alone. Percent specific cytotoxicity is determined by the formula:  $(\text{cpm}_{\text{test}} - \text{cpm}_{\text{spontan}}) \times 100 / (\text{cpm}_{\text{total}} - \text{cpm}_{\text{spontan}})$ , where spontaneous release is determined by incubation of target cells in medium alone (the test substances did not affect spontaneous release), and total release estimated by direct counting of a sample of labelled target cells. Spontaneous release and actual cytotoxicity at effector:target ratio 50:1 of the various target cells: K562: <10, 32-92; CCRF-CEM: <10, 41-84; SKW-3: <15, 24-45; S199N-T2: <20, 18-35. ND = not determined. Goat anti-human  $\beta_2\text{m}$  (GaHu $\beta_2\text{m}$ ) serum, IgG, F(ab')<sub>2</sub>-fragments, and F(ab')-fragments, were produced as described by Björck *et al.*<sup>30</sup>. The purified reagents were used in final concentrations that gave the same  $\beta_2\text{m}$ -binding titer in  $\beta_2\text{m}$ -radioimmunoassay as did whole serum 1:20. The GaHu $\beta_2\text{m}$  had a titer of 0.04 mg  $\beta_2\text{m}/\text{ml}$  in double radial immunodiffusion. To check the specificity for  $\beta_2\text{m}$ , GaHu $\beta_2\text{m}$  was passed through a column of CNBr-coupled human  $\beta_2\text{m}$ -Sepharose<sup>31</sup>. The absorbed antiserum contained less than 0.1 % of the original  $\beta_2\text{m}$ -binding activity. The  $\beta_2\text{m}$ -binding activity could be recovered from the affinity column by low pH-elution. The affinity-purified GaHu $\beta_2\text{m}$  was tested at a  $\beta_2\text{m}$ -binding concentration corresponding to whole serum 1:20. Specificity was also ascertained by including excess amounts of purified human  $\beta_2\text{m}$  (30  $\mu\text{g}/\text{ml}$ ) to anti- $\beta_2\text{m}$  containing cultures. NGS = normal goat serum.



IFN- $\alpha$ <sup>6</sup>) for two hours at 37°C, washed twice and used as effector cells in the NK-assay. Anti- $\beta$ <sub>2m</sub> was found to inhibit basal and IFN-boosted NK-activity to the same extent.

To find out whether anti- $\beta$ <sub>2m</sub> operated via the effector or the target cells, respectively, we performed pretreatment experiments of each cell type separately. Table 2 shows that pretreatment of effector cells with anti- $\beta$ <sub>2m</sub> IgG (compared to pretreatment with normal goat IgG) had very small effects. Pretreatment of target cells resulted in a reduction of the subsequent NK-activity to the same extent as when anti- $\beta$ <sub>2m</sub> was added directly to the cultures.

**Table 2:** Effect of anti- $\beta$ <sub>2</sub>-microglobulin-pretreatment of effector cells or target cells on natural killer cell-mediated lysis.

| <u>Experiment</u> | <u>Target</u> | <u>Effectors +</u><br><u>% inhibition of specific lysis</u> | <u>Targets +</u> |
|-------------------|---------------|-------------------------------------------------------------|------------------|
| 1                 | K562          | 18                                                          | 100              |
| 2                 | K562          | 20                                                          | 71               |
| 3                 | K562          | 12                                                          | 67               |
|                   | CCRF-CEM      | 21                                                          | 59               |
| 4                 | K562          | 10                                                          | 65               |
|                   | CCRF-CEM      | 15                                                          | 63               |

Effector cells (PBL) or target cells were preincubated with GaHu $\beta$ <sub>2m</sub>-IgG (0.75 mg/ml) for 30 min at room temperature (22°C), washed once in complete medium and used in the NK assay at an effector:target cell ratio 50:1 as described in the legend to Table 1. Control cells were treated in an identical way with NG-IgG. Percent inhibition of specific lysis is related to treatment with NG-IgG.

A single-cell assay<sup>7</sup> was used to investigate possible effects on target-binding and target-lysis, respectively. This technique involves formation of NK-tumor cell conjugates, plating them in a semi-solid matrix to prevent recycling and then scoring the number of NK cells that bind target cells and the number of dead NK-bound target cells after an incubation period. Fig. 1 demonstrates that anti- $\beta$ <sub>2m</sub> did not affect the number of PBL that formed conjugates with K562 cells. However, the percentage of PBL-K562-conjugates in which the K562-cell was killed was markedly reduced by the presence of anti- $\beta$ <sub>2m</sub>. Also when highly enriched large granular lymphocytes (LGL; known to contain most NK-cells<sup>8</sup>) were used as effector cells, anti- $\beta$ <sub>2m</sub> had a preferential effect on lysis. These findings were also extended to other target cells than K562 (data not shown).

Thus, the results indicate that target cell  $\beta$ <sub>2m</sub> is of importance during the lytic phase of NK cell-mediated target-destruction. NK cells may exert their lytic effect through soluble factors<sup>2, 9</sup>, presumably LT. This has been claimed also for other types of cell-mediated cytotoxicity, e.g., specific T lymphocyte-mediated cytotoxicity<sup>10</sup>, ADCC<sup>11</sup>, and lectin-

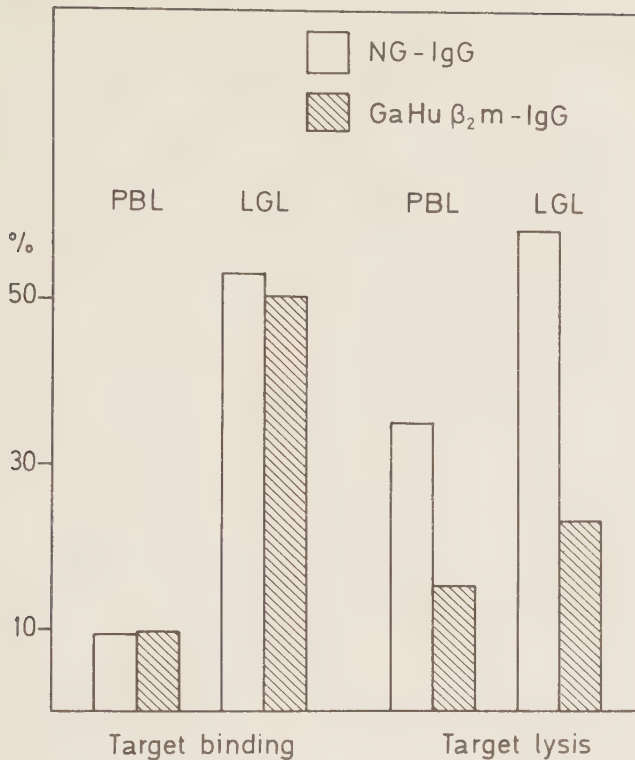


Figure 1

PBL prepared as described in the legend to Table 1 were depleted of adherent cells in autologous serum-coated tissue culture flasks for 1 hour at 37°C. The non-adherent cells were layered on a 5 step discontinuous Percoll<sup>R</sup> (Pharmacia, Uppsala, Sweden) gradient and centrifuged at 550 x g for 30 min at room temperature, as described by Timonen and Saksela<sup>8</sup>. The top layer consisted of 40 % Percoll<sup>R</sup> (v/v) adjusted to 285 mosm by 10x concentrated phosphate-buffered saline (PBS, pH 7.4) and further diluted with 1x PBS. Each 2 ml fraction varied by 2.5 % Percoll<sup>R</sup>. The gradient was loaded with 5 x 10<sup>7</sup> cells. Fraction 2 was enriched for cells with the morphological characteristics of large granular lymphocytes (LGL, 65-75 % in Giemsa-stained preparations) and approximately 80 % stained positively in indirect immunofluorescens tests with the monoclonal HNK-1-antibody<sup>25</sup>. PBL or LGL cells were conjugated with K562 or CCRF-CEM target cells in the presence of NG-IgG or GaHu $\beta_2m$ -IgG (0.75 mg IgG/ml) and plated in agarose as described by Targan et al.<sup>7</sup>. Plates were read immediately to determine the percentage of lymphocytes forming conjugates with target cells (% target-binding; by counting 400 lymphocytes), and after a 3 hour incubation at 37°C in 5 % CO<sub>2</sub>-atmosphere to determine the percentage of bound lymphocytes that were cytotoxic (% target-lysis; by counting 100 conjugates). Before reading, the plates were stained with trypan blue and fixed with 0.5 % formaldehyd in PBS. Three experiments were performed with PBL against K562, two with PBL against CCRF-CEM, two with LGL against K562, and one with LGL against CCRF-CEM. All gave essentially the same results and the figure shows data from one experiment with K562 as target cells.

dependent cell-mediated cytotoxicity<sup>12</sup> (LDCC). Antibodies to LT have been reported to interfere with target cell destruction by various effector cells, including NK-cells<sup>13</sup>. Moreover, the lytic process of NK cells apparently includes a proteolytic step since protease inhibitors suppress NK-mediated killing, highly enriched NK cells produce proteases, and exogenously-added protease potentiate NK-activity<sup>14-17</sup>. In addition, PBL protease activity could be augmented by the major positive NK-regulator IFN, and patients with the Chediak-Higashi syndrome (known to be NK-deficient) have low PBL protease production<sup>17</sup>. Thus, it is conceivable that NK cells operate through a lytic complex - LT - that is activated step-wise by proteolytic events to produce the "lethal hit". This LT-system may be related to the complement-system used by antibodies to produce lysis. If this is the case, it would not be surprising if one or the other of the presumed LT-factors binds to an immunoglobulin-like domain ( $\beta_2m$ ) on the target cell surface. This would be in agreement with the binding of the first complement factor to the Fc-fragment of target-bound immunoglobulins. Phylogenetic studies suggests that NK cells are among the most primitive cells with cytotoxic activity and their wide-spread manifestation points to a high degree of conservation<sup>18</sup>. The killer mechanism would thus most likely involve molecules with a similar degree of conservation, a criterion apparently fulfilled by  $\beta_2m$ <sup>19</sup>.

As killer cells other than NK appear to use an LT-system<sup>10-12</sup>, the above-outlined model might also be valid for these kinds of cell-mediated cytotoxicity. Indeed, Goding & Walker<sup>20</sup> postulated that lysis mediated by cytotoxic T lymphocytes (CTL) is executed in such a way. Anti- $\beta_2m$  inhibits influenza virus-specific<sup>21</sup>, anti-H-2<sup>22</sup>, or anti-H-Y-CTL<sup>22</sup>. In view of the  $\beta_2m$ -H-2-association, the authors of these reports assume that anti- $\beta_2m$  affects target recognition. We suggest that the antibodies may also interfere with the lytic phase. In this context, it is interesting to note that most of the so-called "medial" histocompatibility antigens, that can be targets for unrestricted CTL, are thought to be associated with  $\beta_2m$ <sup>23</sup>.

In conclusion, these results show that the cytotoxic activity of human NK cells is inhibited by antibodies to  $\beta_2m$ . The evidence suggests that this inhibition is due to blocking of the lytic phase at the target cell level. This indicates involvement of  $\beta_2m$ , either in target-mediated activation of the NK cells, or in the transfer of the lytic signal. The authors favour the latter hypothesis. It is thus postulated that  $\beta_2m$  or a closely associated structure serves as acceptor site for a lytic message from NK cells, perhaps delivered by LT.

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*... Und I'm Learning Chinese,  
says Werhner von Braun.*

Tom Lehrer









